

20030121058

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## AD-A171 857 REPORT DOCUMENTATION PAGE

2a. SECURITY CLASSIFICATION AUTHORITY			1b. RESTRICTIVE MARKINGS		
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE			3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited		
4. PERFORMING ORGANIZATION REPORT NUMBER(S) FIN-82-85			5. MONITORING ORGANIZATION REPORT NUMBER(S)		
6a. NAME OF PERFORMING ORGANIZATION University of Maryland School of Medicine		6b. OFFICE SYMBOL (if applicable)	7a. NAME OF MONITORING ORGANIZATION SEP 17 1986		
6c. ADDRESS (City, State, and ZIP Code) 660 W. Redwood St. Baltimore, Maryland 21201			7b. ADDRESS (City, State, and ZIP Code)		
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command		8b. OFFICE SYMBOL (if applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-82-C-2188		
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012			10. SOURCE OF FUNDING NUMBERS		
			PROGRAM ELEMENT NO. 61102A	PROJECT NO. 3M1- 61102BS12	TASK NO. AA
			WORK UNIT ACCESSION NO. 048		
11. TITLE (Include Security Classification) Ion Channels from Mammalian Brain and Heart, Incorporated into Planar Lipid Bilayers: Regulation by Membrane Potential, Calcium, and Neurotoxins					
12. PERSONAL AUTHOR(S) Bruce K. Krueger, Ph.D., Robert J. French, Ph.D.					
13a. TYPE OF REPORT Annual and Final Rpt.		13b. TIME COVERED FROM 82/8/1 TO 85/1/31		14. DATE OF REPORT (Year, Month, Day) 1985 March	
15. PAGE COUNT 67					
16. SUPPLEMENTARY NOTATION (Annual Report covers August 1, 1984 through January 31, 1985)					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP	Membranes, ion channels, single channels, sodium, calcium, electrophysiology saxitoxin, tetrodotoxin, batrachotoxin, neurotoxins, trimethyloxonium, Gonyalux, red tide		
06	15				
06	03				
19. ABSTRACT (Continue on reverse if necessary and identify by block number) Sodium and calcium channels from rat brain membranes, have been incorporated into artificial planar phospholipid bilayer membranes and characterized electrophysiologically. Currents through single channels were studied. Sodium channels were activated by batrachotoxin and were blocked by saxitoxin (STX) and tetrodotoxin (TTX). Block by STX and TTX was increased by membrane hyperpolarization. Chemical modification of the STX binding site eliminated block by STX and TTX, reduced single channel sodium currents, and dramatically eliminated block of the channels by calcium ions. Calcium channels were voltage-dependent with depolarizing potentials favoring channel opening. The single calcium channels were selective for divalent cations over monovalent cations and anions. The selectivity sequence for divalent cations, as measured by single channel conductance, was Ba>Ca>Sr>Mn. There was a direct relationship between the transit time for an ion in the channel (the reciprocal of the single channel conductance) and the probability of channel closing (the mean single channel open dwell time) suggesting that only unoccupied					
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION Unclassified		
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia Miller			22b. TELEPHONE (Include Area Code) 301/663-7325		22c. OFFICE SYMBOL SGRD-RMS

## 19. Abstract (Cont)

channels can close. The data also suggest that there are at lease two (and possibly three) binding sites for calcium ions in the channel pore. ↑

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Report Number FIN-82-85

Ion Channels from Mammalian Brain and Heart, Incorporated into Planar Lipid  
Bilayers: Regulation by Membrane Potential, Calcium, and Neurotoxins.

Annual and Final Report

Bruce K. Krueger, Ph.D.  
Robert J. French, Ph.D.

March 1985

Supported by:

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
Fort Detrick, Frederick, Maryland 21701-5012

Contract No: DAMD17-82-C-2188

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660 West Redwood Street, Baltimore, Maryland 21201

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## SUMMARY

Sodium channels and calcium channels from rat brain membranes, have been incorporated into planar phospholipid bilayer membranes and characterized electrophysiologically. Currents through single channel molecules (single channel currents) were studied. The sodium channels were activated by the neurotoxin batrachotoxin, and were selective for sodium over potassium, cesium, and chloride. They opened as the membrane was depolarized, and were blocked by nanomolar concentrations of the neurotoxins saxitoxin (STX) and tetrodotoxin (TTX). The single channel conductance was 30 pS in symmetrical 0.5 M NaCl, 0.1 mM CaCl<sub>2</sub>. Block of single sodium channels by STX was found to be dependent on the membrane potential with depolarizing potentials reducing the potency of STX block by as much as 50-fold. Both blocking and unblocking rate constants were affected by the membrane potential: depolarization decreased the rate (probability) of channel block by STX and increased the rate (probability) of unblock. These sodium channels are responsible for depolarizing phase of the action potential in nerve and muscle cells and appear to constitute the sole site of action of STX and TTX. Sodium channels in planar bilayers are blocked by both external and internal calcium ions. Block by external calcium appears to be competitive with sodium suggesting binding to a common site. Block by external calcium is also voltage-dependent with hyperpolarizing potentials favoring block. Some of the effects of external calcium may be due to binding at the STX binding site since treatment with trimethylxonium (a carboxyl modifying reagent) eliminates STX block (and binding of <sup>3</sup>H-STX), reduces the degree of calcium block, and reduces the single channel conductance by 30%. STX and calcium protect against modification by TMO. Single calcium channels from rat brain membrane vesicles were also incorporated into planar bilayers. The calcium channels were selective for calcium, barium, and strontium over monovalent cations and anions. The single channel conductances for Ca<sup>++</sup>, Ba<sup>++</sup>, and Sr<sup>++</sup> were 5 pS, 8.5 pS, and 5 pS, respectively, in symmetrical 0.25 M Me<sup>++</sup>Cl<sub>2</sub>. Membrane depolarization increased the probability of channel opening and decreased the probability of channel closing. There was an apparent reciprocal relationship between the single channel conductance and the mean open lifetime for the three permeant cations tested, suggesting a possible relationship between channel gating (voltage dependence) and ion permeation. These calcium channels may be the pathways for calcium entry during stimulus-coupled release of neurotransmitter at synapses in the central nervous system.

## FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

## TABLE OF CONTENTS

Report Documentation Page	
Title Page	1
Abstract	3
Foreword	5
Table of Contents	7
Experimental Results	
A. Specific Aims of Last Proposal	9
B. Publications and Scientific Meetings	10
C. Sodium Channels in Planar Bilayers	12
D. Voltage-dependent Block of Sodium Channels by Saxitoxin and Tetrodotoxin	13
E. Chemical Modification of STX Binding Site	15
F. Block of Sodium Channels by Calcium	34
G. Calcium Channels from Rat Brain in Planar Lipid Bilayers	65
Literature Cited	67
Distribution List	71

## EXPERIMENTAL RESULTS

### A. SPECIFIC AIMS OF LAST PROPOSAL.

In this section of the report we summarize those Specific Aims of our initial proposal (for 1 August 1982 to 31 January 1984) and for the revised proposal (1 February 1984 to 31 January 1985) that have been experimentally addressed during the term of this contract. Some specific aims of the original proposal were dropped as explained in the Annual Reports September, 1983 and September, 1984.

1. To explore [several] aspects of the molecular nature of the interaction of the neurotoxins saxitoxin (STX) and tetrodotoxin (TTX) with sodium channels from mammalian central nervous system.

During the first year of the project, we developed the capability to study and characterize voltage-dependent sodium channels incorporated into planar bilayers (Krueger et al., 1983). In the second year we expanded this preliminary study to include the details of toxin block and ion permeation through the channels. In particular both STX and TTX were found to block in a voltage-dependent manner. Sodium permeation through the channels, block of sodium permeation by divalent cations, and the consequences of modification of the STX/TTX binding site have been investigated. The results are summarized in sections D - F (French et al., 1984).

2. To describe [several] properties of voltage-dependent calcium channels from rat brain.

We have identified and characterized voltage-dependent calcium channels in planar bilayers exposed to rat brain membrane vesicles. The results are summarized in section G (Nelson et al., 1984; Nelson, 1984; 1986).

3. To reconstitute and study sodium channels and calcium channels from mammalian heart sarcolemma in planar bilayers and to compare their properties with those of sodium and calcium channels from brain.

In preliminary experiments, we have tentatively identified STX/TTX sensitive sodium channels as well as a novel, rectifying cation channel in planar bilayers exposed to rat heart sarcolemma membranes. To date no evidence for heart calcium channels has been found. No additional progress has been made on these cardiac channels as a consequence of our concentrating on the sodium and calcium channel studies described above.

## B. PUBLICATIONS AND SCIENTIFIC MEETINGS.

Work under this contract has resulted in the following publications:

- Krueger, B.K., J.F. Worley, III, and R.J. French. Single sodium channels from rat brain incorporated into planar lipid bilayer membranes. Nature 303: 172-175 (1983).
- French, R.J., J.F. Worley, III, and B.K. Krueger. Voltage-dependent block by saxitoxin of sodium channels incorporated into planar lipid bilayers. Biophysical Journal 45: 301-310 (1984).
- Nelson, M.T., R.J. French, and B.K. Krueger. Single calcium channels from rat brain in planar lipid bilayers. Nature 308: 77-80 (1984).
- Nelson, M.T. Single calcium channels from rat brain in planar lipid bilayers. in "Epithelial Calcium and Phosphate Transport: Molecular and Cellular Aspects" in Progress in Clinical and Biological Research, Vol. 168, (F. Bronner & M. Peterlik, eds.) A. Liss, New York: 59-64 (1984).
- Worley, J.F. Sodium channels in planar lipid bilayers: Effects of neurotoxins, divalent cations, and chemical modification on ion permeation. Ph.D. Thesis. (1985)
- Nelson, M.T. Interactions of divalent cations with single calcium channels from rat brain synaptosomes. J. Gen. Physiol. 87: 201-222 (1986).
- French, R.J., B.K. Krueger, and J.F. Worley. From brain to bilayer: Sodium channels from rat neurons incorporated into planar lipid bilayers. From: "Ionic Channels in Cells and Model Systems", R. Latorre, ed., 273-290 (1986).
- French, R.J., J.F. Worley, M.B. Blaustein, W.R. Romine, K. Tan, and B.K. Krueger. Gating of Batrachotoxin-activated Sodium Channels in Lipid Bilayer Membranes. in "Ion Channel Reconstitution", C. Miller, ed., 363-383 (1986).
- The following abstracts have resulted from work on this project:
- Nelson, M.T. and R. Reinhardt. Incorporation of potassium channels from rat brain synaptosomes in planar bilayers. Biophys. J. 41: 36a (1983).
- French, R.J., J.F. Worley, and B.K. Krueger. Unitary current fluctuations due to saxitoxin block of sodium channels in planar bilayers. Biophys. J. 41: 142a (1983).
- Krueger, B.K., J.F. Worley, and R.J. French. Sodium channels from rat brain in planar lipid bilayers. Biophys. J. 41: 142a (1983).
- Nelson, M.T. Permeant ions affect the conductance and open times of single calcium channels from rat brain. J. Physiol. 357: 58P (1984).
- Lederer, W.J. and M.T. Nelson. Single Na-K channel current measurements from calf heart muscle. J. Physiol. 357: 46P (1984).



- Worley, J.F. Sodium channels in planar lipid bilayers: Effects of divalent cations on ion permeation. Biophys. J. 45: 186a (1984).
- Nelson, M.T. Single calcium channel current measurements from brain synaptosomes in planar lipid bilayers. Abst. Soc. Neuroscience. 9: 508 (1983).
- Nelson, M.T. Reduction of single calcium channel currents by lanthanum and cadmium. Biophys. J. 45: 395a (1984).
- Nelson, M.T., R. Reinhardt, and W.J. Lederer. Sodium channels from mammalian heart muscle incorporated into planar lipid bilayers. Biophys. J. 45: 185a (1984).
- Nelson, M.T. and R. Reinhardt. Single ion channel current measurements from rat brain synaptosomes in planar lipid bilayers. Biophys. J. 45: 60-62 (1984).
- Krueger, B.K., J.F. Worley, M.T. Nelson, M.B. Blaustein, and R.J. French. The planar bilayer: Studying structure and function of ion channels. "Transactions of the American Society for Neurochemistry" 16: 318 (1985).
- French, R.J., M.B. Blaustein, W.O. Romaine, K. Tam, J.F. Worley, and B.K. Krueger. Voltage-dependent gating of single batrachotoxin-activated sodium channels in planar bilayers. Biophys. J. 47: 191a (1985).
- Nelson, M.T., J.F. Worley, T.B. Rogers, and W.J. Lederer. Magnesium blockade of a cation-selective rectifying channel from heart muscle. Biophys. J. 47: 143a (1985).
- Worley, J.F., R.J. French, and B.K. Krueger. Interactions of divalent cations with single sodium channels. Biophys. J. 47: 439a (1985).
- Nelson, M.T. Divalent cation interactions with single calcium channels from rat brain: Evidence for two sites. Biophys. J. 47: 67a (1985).

Staff members of this project attended the following meetings to present the experimental results from this project:

B.K. Krueger and R.J. French. Gordon Research Conference on "Ion Channels in Muscle and Other Excitable Tissues", 8/82.

R.J. French, M.T. Nelson, J.F. Worley, Biophysical Society Meeting, San Diego, CA, 2/83.

B.K. Krueger (invited speaker) Gordon Research Conference on "Molecular Pharmacology", 6/83.

R.J. French (invited speaker) Gordon Research Conference on "Cell Membranes", 8/83.

R.J. French (invited speaker) XXIXth International Physiological Congress, Sydney, Australia, 8/83.

R.J. French (presented plenary paper), B.K. Krueger, J.F. Worley, and M.T. Nelson, invited participants in 4th Biophysical Discussions on Ion Channels, Airlie, VA, 10/83.

M.T. Nelson, 13th Annual Meeting of the Society for Neuroscience, Boston, MA, 11/83.

R.J. French, M.T. Nelson, J.F. Worley. Biophysical Society Meeting, San Antonio, TX, 2/84.

M.T. Nelson, (invited speaker), Symposium on "Calcium and Phosphate Transport across Biomembranes" Vienna, Austria, 3/84.

M.T. Nelson, meeting of the Physiological Society, Cambridge, UK, 7/84.

J.F. Worley, Attended (with fellowship) Cold Spring Harbor course on "Advanced Electrophysiological Techniques", 7/84.

R.J. French (invited speaker) and B.K. Krueger. Gordon Research Conference on "Ion channels in muscle and other excitable membranes", 8/84.

R.J. French, Invited lecturer at "International School on Ionic Channels" Santiago, Chile, 11/84.

R.J. French, B. K. Krueger, J.F. Worley, M.B. Blaustein, M.T. Nelson. 29th Annual Meeting of the Biophysical Society, Baltimore, MD, 2/85.

B.K. Krueger, (invited speaker) Symposium on "Ion Channels in Bilayers". Sixteenth Annual Meeting of the American Society for Neurochemistry, Baltimore, MD, 3/85.

### C. SODIUM CHANNELS IN PLANAR BILAYERS.

During the first year of this contract, voltage-dependent sodium channels from rat brain membranes (1) were incorporated into planar phospholipid bilayer membranes (2). The channels were activated by batrachotoxin (BTX) on the side opposite rat brain membrane addition (trans) and were blocked by saxitoxin (STX) from the side of vesicle addition (cis). This allowed the assignment of the cis side as the outside of the bilayer with respect to the channels. Both macroscopic (multichannel) and single channel currents were studied. The single channel conductance was 30 pS ( $30 \times 10^{-12} \text{ ohm}^{-1}$ ) in symmetrical 0.5 M NaCl. STX blocked with an apparent dissociation constant of about 4 nM. Stepwise, unitary current fluctuations were observed which were due to the blocking and unblocking of individual sodium channels. Single BTX-activated sodium channels were selective for sodium over potassium, cesium, and chloride. Hyperpolarization favored channel closing. Block of single sodium channels by STX was voltage-dependent with hyperpolarizing potentials favoring block. There is little doubt that these channels are identical to those that are responsible for the depolarizing phase of the action potential of nerve cells and that are the only known site of action of STX and TTX. Details of these results can be found in Krueger et al., 1983 (2). Several particularly interesting findings came out of this work. This was the first report in the literature of the successful incorporation of voltage-dependent sodium channels into an artificial membrane. By taking advantage of the fact that in the presence of BTX, the channels are nearly always open at

membrane potentials of  $\pm 60$  mV, we demonstrated stepwise, unitary current fluctuations due to the blocking and unblocking of individual sodium channels by STX. This demonstrated for the first time that block by STX is all-or-none, that is, a channel is either fully open or completely blocked by STX. Another interesting result that is described in more detail in French et al., 1984 (3) is that block by STX is affected by the membrane potential with depolarizing potentials reducing the potency of block by up to 50-fold.

All experiments on sodium channels reported here have been carried out with channels incorporated into planar bilayers formed from solutions of phospholipids (either phosphatidylethanolamine or phosphatidylethanolamine and phosphatidylserine) in decane as described in Krueger et al., 1983 (2). Membrane vesicles, prepared from homogenates of rat brain, are added to one side of the planar bilayer (the cis side) and channel incorporation monitored by observing stepwise single channel current fluctuations with a potential applied to the membrane. Unless otherwise specified, the solutions on both sides of the planar bilayer contained identical electrolyte compositions (usually 500 mM or 250 mM NaCl) and batrachotoxin (BTX) was present on the side opposite vesicle addition (the trans side). Ionic currents through single channels was recorded using either a Yale Mark IV patch clamp or one of several homemade devices constructed in our electronics shop. With the membranes used in these studies (normally 0.25 mm in diameter), we can record currents steps of about 1 pA at 500 Hz or about 0.2 pA at about 100 Hz. These bandwidths place limitations on the resolution of fast gating events and we are working on methodologies to reduce the membrane size in order to increase the effective recording bandwidth.

#### D. VOLTAGE-DEPENDENT BLOCK OF SODIUM CHANNELS BY SAXITOXIN AND TETRODOTOXIN.

Summary. Our preliminary finding (2) that the potency of block of sodium channels by STX varied with the membrane potential has now been investigated in detail. All experiments were conducted on bilayers containing only one or a few sodium channels. The voltage dependence of STX block was evaluated from steady state measurements of the fractional block as a function of STX concentration over a range of membrane potentials. It was found that the dissociation constant ( $K_I$ ) for STX block ranged from about 1 nM at -60 mV (near the normal resting potential) to about 50 nM at +60 mV. By evaluating the kinetic parameters of unitary single channel fluctuations induced by STX, we were able to determine that depolarization decreased the blocking rate constant and increased the unblocking rate constant. At each potential, the  $K_I$  derived from kinetic parameters agreed well with the  $K_I$  obtained from steady state determinations.

Voltage-dependent STX block was entirely unexpected. Several investigators had previously considered the possibility that STX or TTX block was voltage-dependent (4-6) but in each case no evidence could be found to demonstrate such an effect of voltage on block. One report (7) suggested that depolarization of heart cells increased the potency of TTX block, however, the validity of the experimental methods used to carry out those experiments have been questioned (8), and more recent studies have failed to confirm that finding (9). As summarized above, our results demonstrate that depolarization reduces the potency of STX block at least under the specific experimental conditions used in our experiments.

A voltage-dependent process suggests that one or more electric charges are interacting with the membrane electric field. The most likely candidate for

those charges is the STX molecule itself which is a divalent cation under physiological conditions. One possible mechanism is that the binding site for STX lies inside the channel at a location within the membrane electric field. Thus block by STX, approaching from the outside, would be favored by negative-inside (hyperpolarizing) potentials, making the potency of block greater (10,11). Our initial results appeared to be consistent with this model. However, we have subsequently compared the action of TTX, a monovalent cation, with the divalent STX. The  $K_I$ 's for these two toxins vary identically with voltage, changing e-fold for a 40 mV change in voltage (Figure 1). Since the two charges on STX are separated by only 3-4 Å, it is highly unlikely that one of these charges could penetrate into the transmembrane electric field without the other. Given identical effects of voltage on the action of the toxins with charges of +1 and +2, the voltage-dependence is unlikely to result from entry of the toxin molecules into the transmembrane field. The voltage dependence must arise from some common, voltage-dependent step in the binding and blocking reactions, perhaps a voltage-dependent conformational change in the channel protein itself.

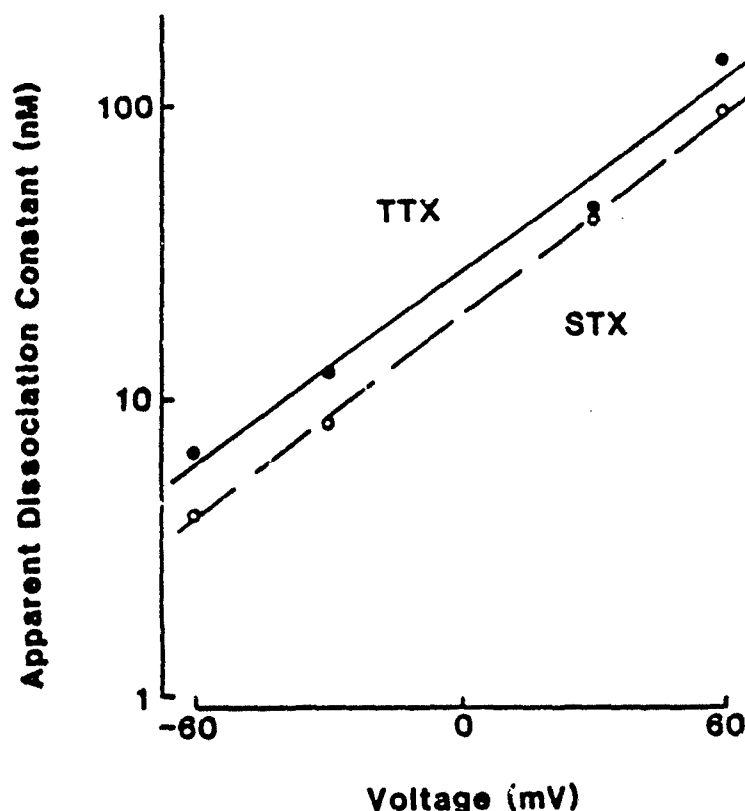


Figure 1. The apparent dissociation constants ( $K_d$ 's) for TTX (filled circles) and STX (open circles) are plotted as a function of membrane potential.  $K_d$ 's were calculated from the relation  $K_d = [TX]/(fb^{-1} - 1)$  where fb (fraction blocked) was determined from single sodium channel current measurements in planar bilayers.

### E. Chemical Modification of the STX Binding Site.

Many lines of evidence suggest that an ionized carboxyl group located on the sodium channel receptor is intimately involved in saxitoxin and tetrodotoxin binding and block. Radiolabeled toxin binding experiments using membrane vesicles (12) and intact excitable membranes as well as electrophysiological recordings (13) have demonstrated involvement of an acidic site ( $pK_a$  about 5) in toxin binding and block. These results suggest that a carboxyl or phosphate group might be intimately associated with the toxin receptor.

Modification of sodium channels by carbodiimide or trimethylxonium tetrafluoroborate (TMO), two carboxyl side chain modifiers, have been shown to render sodium channels insensitive to TTX (14). TMO, a more specific and selective chemical modifier than carbodiimide, has been shown to inhibit toxin binding to sodium channels irreversibly (12). In addition, Sigworth and Spalding (15) and Spalding (16) demonstrated that sodium currents in frog skeletal muscle are insensitive to tetrodotoxin after TMO modification. They also found that, following TMO modification, sodium currents were reduced, ion selectivity, as determined from alterations in the sodium current reversal potential in the presence of organic cations, was unaffected, and the sensitivity of sodium currents to alterations in pH was reduced. These results demonstrated that the toxin receptor site is distinct from the domains of the sodium channel responsible for ion selectivity and gating. This section reports the effects of chemical modification, by TMO, on sodium channels from rat brain incorporated into planar lipid bilayers. Chemical modification was performed by the addition of TMO to the extracellular surface of sodium channels incorporated into lipid bilayers. For  $^3H$ -STX binding experiments, TMO was added to the membrane vesicle preparation (P3). Sodium channels in the bilayer were characterized electrically, before and after TMO treatment.

### Effect of TMO modification on single sodium channel properties

Figure 2 illustrates the modification of single batrachotoxin-activated sodium channels by TMO. Sodium channels from rat brain were incorporated into membranes composed of PE with the identical solution composition on both sides to minimize asymmetries in membrane surface charge. Figure 2A shows a normal sodium channel which displays many transitions between the open and closed states as shown by the brief closing events lasting up to a few tens of milliseconds, many of which are too brief to be resolved at the present recording bandwidth (500 Hz). These closing events reflect the voltage-dependent openings and closings of BTX-activated channels. At -60 mV the channel remains open most of the time (greater than 98%). These channels were selective for sodium over potassium, and are sensitive to STX and TTX (2). Upon addition of STX (5 nM) to the extracellular side, long lived "closing" events due to toxin block are observed. Note that the single channel conductance before and after toxin addition is unchanged as shown by French, Worley, and Krueger (3). Toxin block is clearly resolved as an all-or-none event, in contrast to the effect of less potent blockers like calcium. These channels also display voltage dependent STX and TTX block in which depolarizing potentials favor an unblocked state (3,21). These results are consistent with toxin binding to a site located approximately 30-40% of the electrical distance of the transmembrane field from the extracellular surface.

Figure 2B illustrates the consequences of TMO modification. Following incorporation into neutral (PE) membranes, the channels were first characterized with respect to their single channel conductance and kinetic behavior to verify normal sodium channel function. After TMO addition (50-60 nM), the single channel current at the same applied potential (-60 mV) was reduced by 37%. Moreover, STX failed to block the channels at a concentration (50 nM) which would normally block unmodified sodium channels by over 96% at this potential. TMO modified sodium channels were also insensitive to TTX (100 nM). I have not determined whether toxin binding is completely lost or the affinity is greatly reduced, however, the affinity for toxin would have to be decreased by at least two orders of magnitude to account for this result.

Figure 3 illustrates the single channel current-voltage relationship before and after TMO modification. Note that the relationships are linear over the voltage range studied and that TMO lowers the single channel conductance from  $25 \pm 0.6$  pS to  $15.8 \pm 0.3$  pS. Hydrogen ions are produced when TMO reacts with water; one hydrogen ion is generated per molecule of TMO. However, the linearity of the current-voltage relationship shows that the buffering was adequate to keep the pH constant. If the extracellular pH had not been maintained, then inward single channel current would have been reduced by the presence of hydrogen, in a voltage-dependent manner.

#### Reduction of single channel conductance and toxin sensitivity

When STX (1-10 nM) was added before TMO addition, alterations in channel function were not observed (n=4 experiments). Similarly, when two or more channels were in the bilayer, occasionally one was modified while the other was not as illustrated in Figure 4. At 60 mV, a toxin unblocked state is favored for normal, unmodified STX sensitive sodium channels in which the  $K_d$  for block is about 80 nM. At -60 mV block is much more potent and the  $K_d$  is about 2-4 nM (3). Figure 4 shows a bilayer containing two sodium channels, only one of which has been modified by TMO. Note that the modified, low conductance sodium channel is always unblocked and the single channel current is about 0.9 pA. However, the unmodified sodium channel which is blocked and unblocked by STX has a single channel current of about 1.4 pA. If one were to extend this record to much longer times the modified channel would never display the characteristic long lived blocked and unblocked states displayed by the unmodified channel in this bilayer. At -60 mV, the unmodified sodium channel is initially unblocked and then blocked by STX very soon after the voltage step and does not become unblocked again. On the other hand, the TMO modified channel is never blocked by STX. This result, and others, (n=3) demonstrate that when a sodium channel was modified by TMO, both the reduced single channel conductance state and toxin insensitivity were observed and suggested that both of these physiological consequences of TMO resulted from modification of a common site.

#### TMO reaction with water

A concern in these studies was that the by-products of the TMO reaction with water (methanol and ether) were producing changes in sodium channel properties rather than the reagent itself. To test this, TMO was allowed to react completely with solutions bathing a preformed bilayer prior to addition of

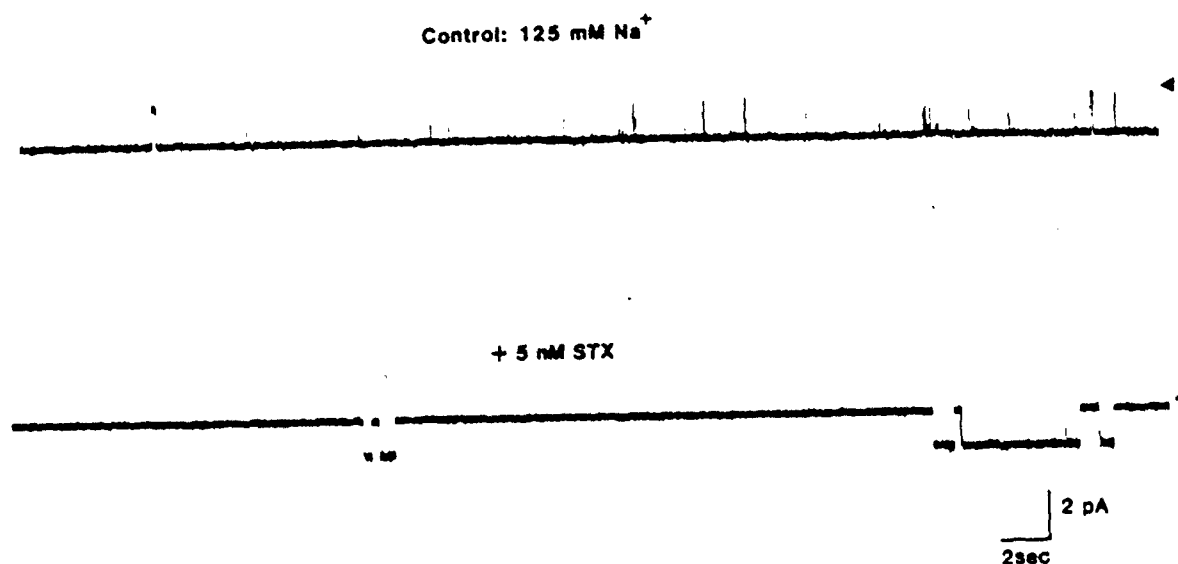


Figure 2A. A single sodium channel was incorporated into a neutral (PE) membrane in which the solutions on both sides of the bilayer contained 125 mM sodium. The current fluctuations were recorded at -60 mV and the zero current levels are indicated by an arrow so that upward current deflections represent channel closings. In the absence of STX, the channel spent over 98% of the time in the open, ion conducting state (top). When 5 nM STX was added to the extracellular side (bottom), the channel was in the closed or blocked state most of the time, with only infrequent openings as the STX molecule dissociated from the blocking site.

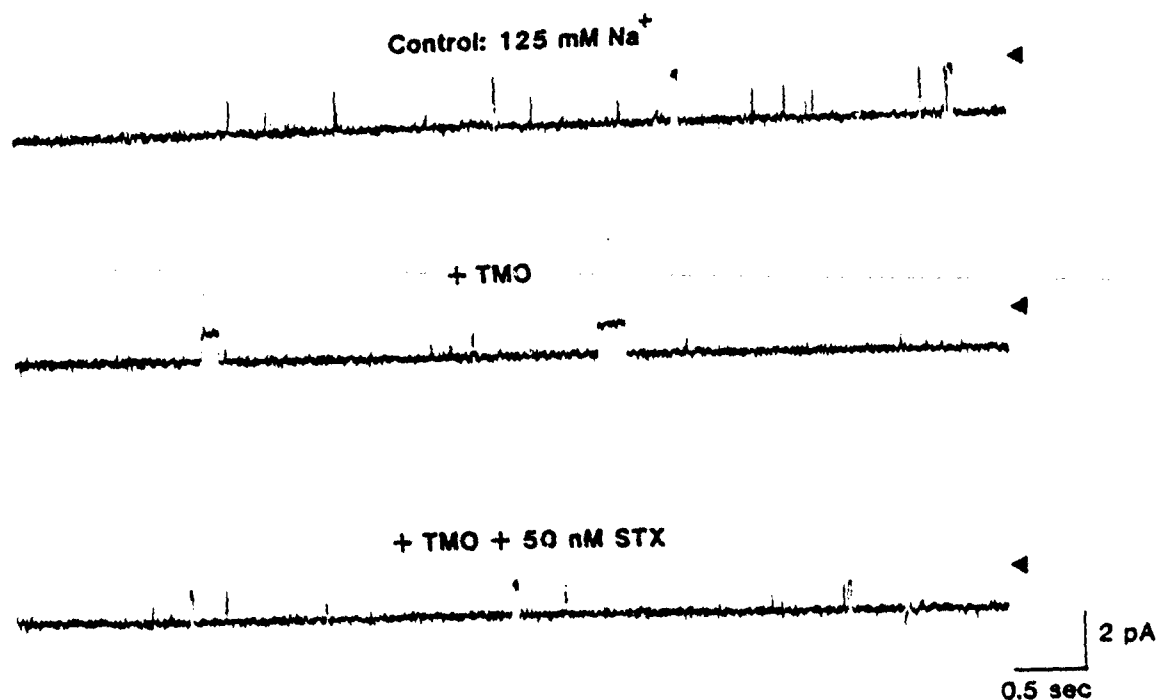


Figure 2B. These current fluctuations are from a different channel under the same conditions as in A except that no STX was present (top current trace). When TMO (50 mM) was added to the extracellular side of the incorporated channel, the single channel current was reduced by 37% (middle). When 50 nM STX was added to the same side as TMO, there were no long lived closing or blocking events as observed in A. The STX concentration was ten times higher in B than in A. Note the difference in time scale for A. and B. These records were filtered at 150 Hz.



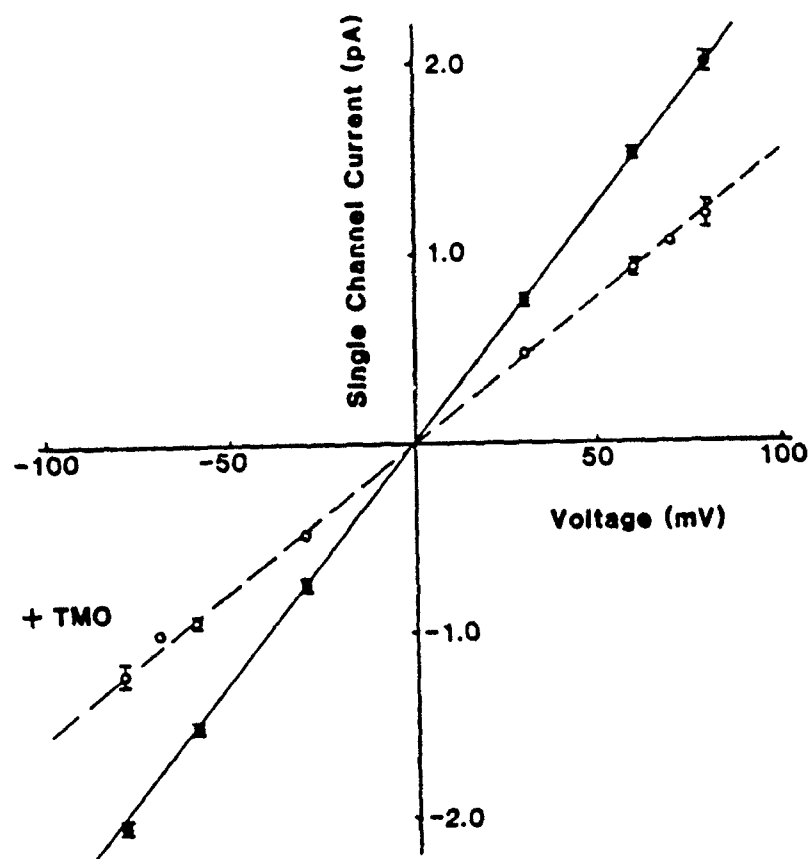


Figure 3. Current-voltage relationships for normal and TMO modified sodium channels. The conductance of sodium channel prior to addition of the chemical modification reagent was  $25 \pm 0.6$  pS (filled circles). In 9 experiments, following TMO modification the single channel conductance is reduced to  $15.8 \pm 0.3$  pS (open circles).

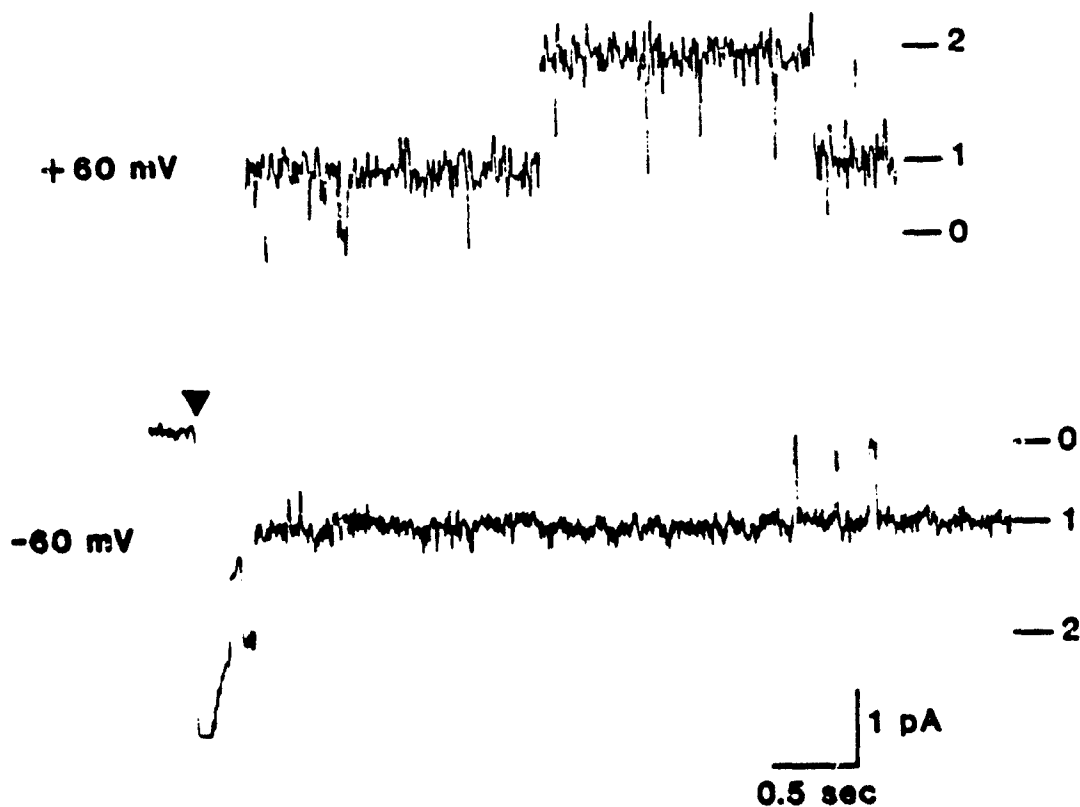


Figure 4. Single channel current fluctuations from a neutral membrane containing an unmodified sodium channel and a TMO modified sodium channel. These records were taken at 60 and -60 mV in the presence of 50 nM STX. For the current record at the bottom the arrow indicates that the potential was changed from 0 mV to -60 mV; note the decay in the capacitive current spike. The open states (or levels) of the two channel types are indicated at the right. The larger conductance site displays voltage-dependent block by STX, while the STX insensitive channel is open most of the time. Note the STX block on the capacitive transient when the voltage is stepped to -60 mV.

biological material. The pH was maintained at 7.0 by the addition of concentrated NaOH. Membrane vesicles (P<sub>3</sub>) were then added, resulting in incorporation of normal, unmodified sodium channels which were STX sensitive and showed no reduction in single channel conductance. Furthermore, no detectable alterations in any other functional properties were observed. This demonstrates that the trimethyloxonium ion is necessary to modify or convert single sodium channels to a reduced single channel conductance state which is toxin insensitive.

In approximately 20% of the attempts to modify sodium channels, toxin insensitive sodium channels were not observed following addition of the carboxyl modifying reagent. Presumably, this was due to the labile nature of the reagent in aqueous solution, particularly at room temperature (about 24°C). The half life of TMO in an aqueous solution is between 1-8 minutes at 50°C and is very temperature dependent (16,17). In successful experiments, solid TMO, which had been preweighed and stored at 4°C no longer than a few hours in a sealed test-tube, was added to the extracellular side of the channel with brisk stirring.

#### Effect of extracellular calcium on single channel currents

Divalent cations have been shown to influence sodium ion movement through sodium channels in a variety of tissues. Figure 5A shows the effect of extracellular calcium addition on single sodium channel currents. Sodium channels were incorporated into bilayer membranes composed of neutral phospholipids (PE) in which the solutions on both sides of the bilayer were symmetrical, and contained less than 100 nM divalents. At -60 mV, the sodium channels were mostly open with occasional fluctuations to the closed state. When 10 mM calcium was added to the extracellular side of an incorporated sodium channel, the single channel current was reduced by about 60%. Notice at the end of the record the current fluctuations cease and the current level indicates that the channel is closed. This is due to the presence of a low concentration of STX (5 nM) which was added to provide an unambiguous determination of the single channel current magnitude. This calcium induced current reduction is presumably due to a rapid movement of calcium ions into and out of a blocking site, located in the sodium channel, producing a time-averaged reduction in the single channel current.

In addition to current reduction, there is an effect of extracellular calcium on the channel's activation kinetics. The sodium channel appears to spend a greater amount of time in the closed, non-conducting state in the presence of extracellular calcium. The effect of extracellular calcium on the kinetic properties of sodium channels has been described previously and was expected.

The single channel current-voltage relationship before and after calcium addition are shown in Figure 5. As the membrane voltage is made more negative, the inward single channel current is reduced (blocked) from control levels by calcium. There is greater current reduction at -90 mV (about 74%) than at -30 mV (about 53%). Therefore, reduction of inward single sodium channel currents by calcium is voltage-dependent, such that more hyperpolarized potentials favor greater current reduction.

Table 1 shows the influence of voltage on the reduction of inward currents by extracellular calcium. The apparent affinity (K<sub>i</sub>) for calcium evaluated at each potential has been calculated from the fraction blocked current (F<sub>b</sub>) ob-

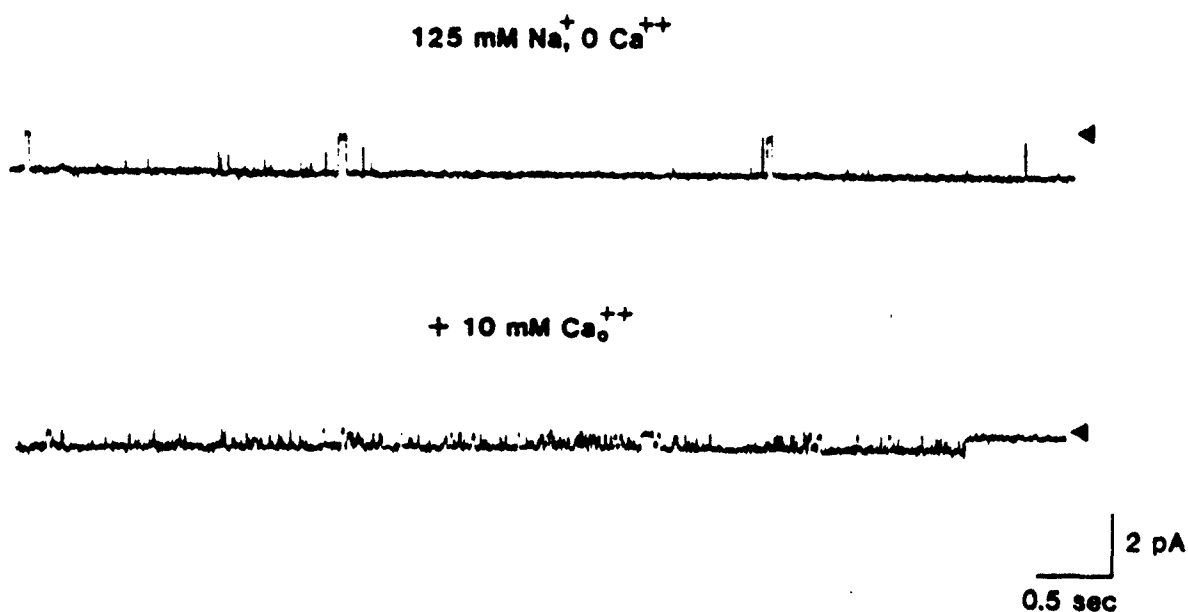


Figure 5A. Effects of extracellular calcium on single sodium channels. The single sodium channel was incorporated into a neutral membrane bathed by symmetrical 125 mM sodium solutions and the zero current levels are indicated by the arrows. Single channel current fluctuations were recorded at -60 mV in the absence (top trace) and presence of 10 mM extracellular calcium (bottom). A small amount of STX was added to the extracellular solution upon addition of calcium and is responsible for the long closing observed at the end of the current record at the bottom.

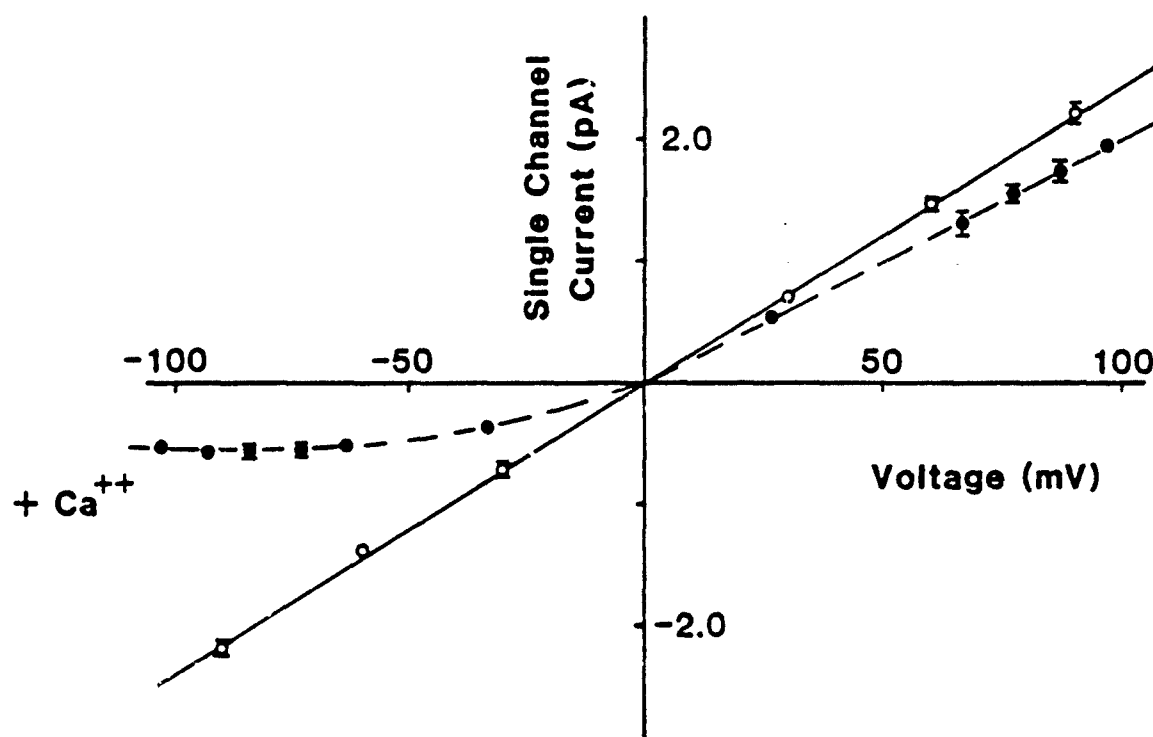


Figure 5B. The current-voltage relationship for sodium channels in the absence (open circles) and presence (closed circles) of 10 mM extracellular calcium. Each point represents data collected from 6 experiments and the smooth curve was drawn by eye.

tained from the following relationship:

$$K_i = (Fb^{-1} - 1) \cdot [Ca^{2+}] \quad \dots (1)$$

The more negative the membrane potential, the higher the apparent affinity for calcium. By adopting the formalism used by Woodhull (10), one can determine the apparent voltage dependence of block, or fraction of the field sensed by calcium, from the following relationship

$$K_i(E) = K_0 \cdot \exp(DzFE/RT) \quad \dots (2)$$

where  $K_i(E)$  and  $K_0$  are the calculated inhibition constants for calcium at the indicated potential (E) and 0 mV, respectively, D is the fraction of the applied electric field sensed by the divalent blocker, z is the valence of the ion, and R, T, and F have their usual meanings. From the fraction of blocked current at each potential the  $K_i$  can be determined from equation 2 (Table 1). These results suggest that calcium binds to a site located approximately 23% of the electrical distance from the extracellular surface and is in close agreement to the results from other studies on extracellular calcium addition (10).

Table 1. Effect of voltage on extracellular calcium block

E (mV)	Fraction of current blocked	$K_i$ (mM)
-33.7	$0.51 \pm 0.04$	$9.6 \pm 1.6$
-63.7	$0.64 \pm 0.03$	$5.6 \pm 0.9$
-73.7	$0.68 \pm 0.03$	$4.7 \pm 0.7$
-83.7	$0.71 \pm 0.02$	$4.1 \pm 0.5$
-93.7	$0.74 \pm 0.02$	$3.5 \pm 0.3$
-103.7	$0.79 \pm 0.02$	$2.6 \pm 0.3$

\* Data point was determined from one membrane. All other values were collected from 4-7 membranes.

#### Effects of calcium on STX block

Since STX is specific for the sodium channel, it may be used as a tool to study interactions of other agents or ions with the channel. Figure 6 illustrates the influence of calcium on STX block of a single sodium channel incorporated into a neutral (PE) membrane with symmetric solutions. STX (10 nM) was added to the extracellular side of the incorporated sodium channel. At -60 mV the channel spends over 95% of its time blocked by STX. When 10 mM calcium was added to both sides of the bilayer, the sodium channel, on the average, spent a longer time in the open, unblocked state as indicated by the increased open (STX unblocked) dwell times (Figure 6). This suggests that the on (or blocking) rate for STX is reduced in the presence of calcium. Furthermore, the overall fraction of time the channel spends in the unblocked state is increased, indicating that the overall sensitivity to toxin has been decreased. Also, the single channel current was reduced about 60% in the presence of 10 mM calcium (see Figure 5 for comparison). Calcium was added symmetrically to avoid a change in the time the channel spends in the closed state due to a shift in the voltage dependence of channel gating as observed in Figure 5A. Therefore, current fluctuations between the open and closed state could be attributed to STX blocking

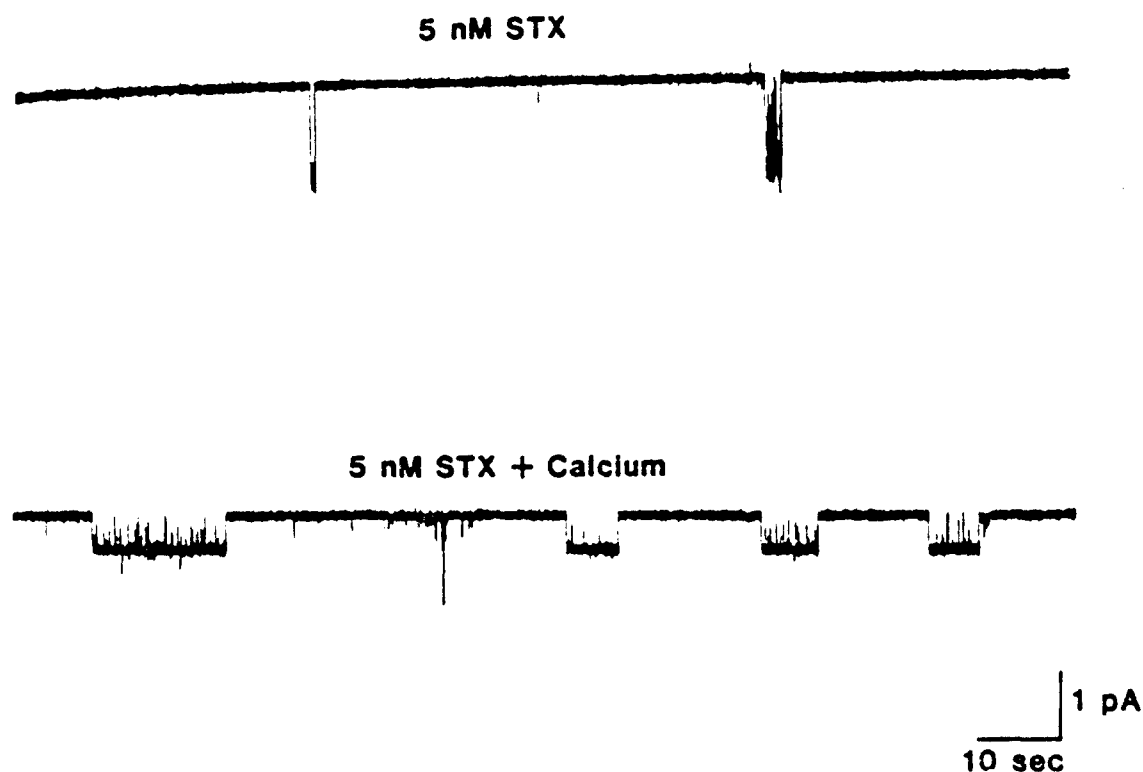


Figure 6. Effect of calcium on STX block. A single sodium channel was incorporated into neutral (PE) planar lipid bilayers with symmetrical 125 mM sodium and 5 nM STX only on the extracellular side. The current fluctuations were recorded at -60 mV and the zero current levels are indicated by the arrows so that downward fluctuations in the current record are channel openings. The current record at the top shows that the channel is blocked by STX over 90% of the time, and becomes unblocked only briefly. When 10 mM calcium is added to both sides of the membrane, the single channel current is reduced the mean unblocked dwell time is increased and the channel spends a larger fraction of the time in the open, unblocked state.

and unblocking events, uncomplicated by alterations in gating behavior. Similar effects on STX block were obtained with calcium addition to the extracellular side alone.

Table 2 shows the results of kinetic and steady state analysis of records such as those of Figure 6. There is about a five-fold reduction in the on (blocking) rate for STX and a small effect on the STX off (unblocking) rate. Overall, calcium causes about a seven-fold increase in the apparent dissociation constant for STX block determined from either kinetic rate constants or steady state fractional block measurements. The extent to which the off rate for STX block was altered in the presence of calcium varied from experiment to experiment. Given the small sample size of single channel current fluctuations between the blocked and unblocked state (about 60), the confidence interval is about 13-20% therefore, one must conclude that the off rate for STX was apparently unaffected by calcium since the range of standard errors of the means overlap, so that their differences are not significant. These results suggest that calcium competes with STX for the blocking site, thereby reducing STX affinity.

Table 2. Calcium influence on STX block.

[Ca <sub>o</sub> <sup>2+</sup> ]	Kon 10 <sup>-8</sup> M <sup>-1</sup> s <sup>-1</sup>	Koff s <sup>-1</sup>	Kd (nM)	
			rate constant	steady state
0 mM	0.310 ± 0.010	0.034 ± 0.020	1.3 ± 0.3	1.5 ± 0.4
10 mM	0.065 ± 0.010	0.058 ± 0.020	9.6 ± 0.4	9.8 ± 0.6

These determinations were collected from three separate single channel membranes.

#### Calcium inhibits <sup>3</sup>H-STX binding

<sup>3</sup>H-STX binding to membrane vesicles (P<sub>3</sub>) is also reduced in the presence of calcium as shown in Figure 7. The double reciprocal plots illustrate a shift in the K<sub>d</sub>, without altering V<sub>max</sub> for binding. In these experiments, 10 mM calcium caused a 5-6 fold increase in the dissociation constant for <sup>3</sup>H-STX binding, with the characteristics of a competitive inhibitor. These values are similar to those obtained from <sup>3</sup>H-STX binding experiments under similar conditions. Therefore, it appears that calcium can compete for the STX binding and blocking site.

#### Calcium and STX protect against TMO modification

Competition between calcium and STX could be a manifestation of interactions of the blockers with the sodium channel at separate sites. However, Figure 8 illustrates that both STX and calcium are able to protect against TMO modification, presumably by binding to the toxin binding site. The reduction in the number of chemically modified STX binding sites occurs in a dose-dependent manner which closely follows the potency for block by STX and calcium obtained from single channel measurements. As the STX or calcium is increased, the number of <sup>3</sup>H-STX binding sites recovered after TMO modification increases. The arrows indicate the predicted occupancy of the ion, STX or calcium, as determined from their effects on the single sodium channels in the bilayer. The parallel between the sensitivity, or potency of channel blockade by calcium and STX and their



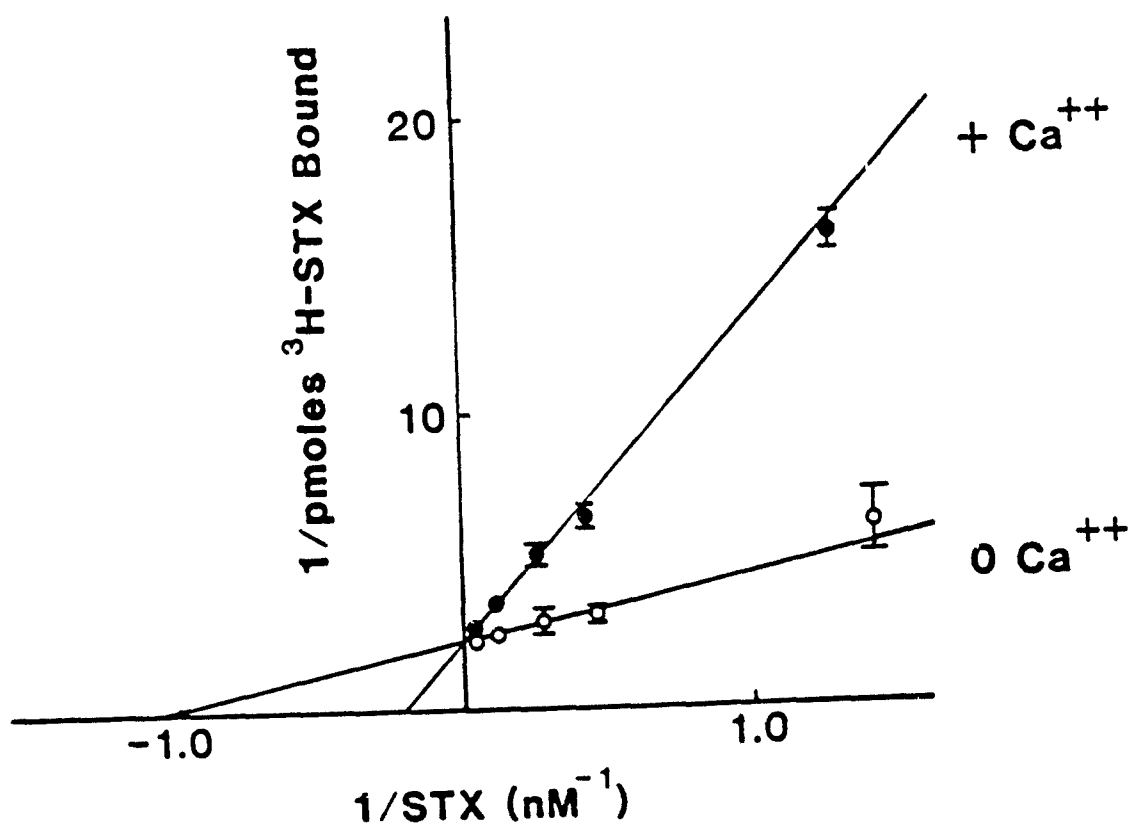


Figure 7. Double reciprocal plot of  $^3\text{H}$ -STX binding to membrane vesicles ( $\text{P}_3$ ). Binding was measured under equilibrium conditions at  $40^\circ\text{C}$  in the presence of 125 mM sodium at pH 7.0 with (closed circles) and without (open circles) 10 mM calcium. Each point represents four determinations of radiolabeled STX bound to membrane vesicles (0.2  $\mu\text{g}/\text{ml}$ ) at the indicated concentration. In the presence of calcium, the STX affinity is reduced. From double reciprocal plots such as this one, calcium increased the apparent dissociation constant from  $0.87 \pm 0.06$  nM to  $5.12 \pm 0.04$  nM  $^3\text{H}$ -STX ( $n=3$ ).

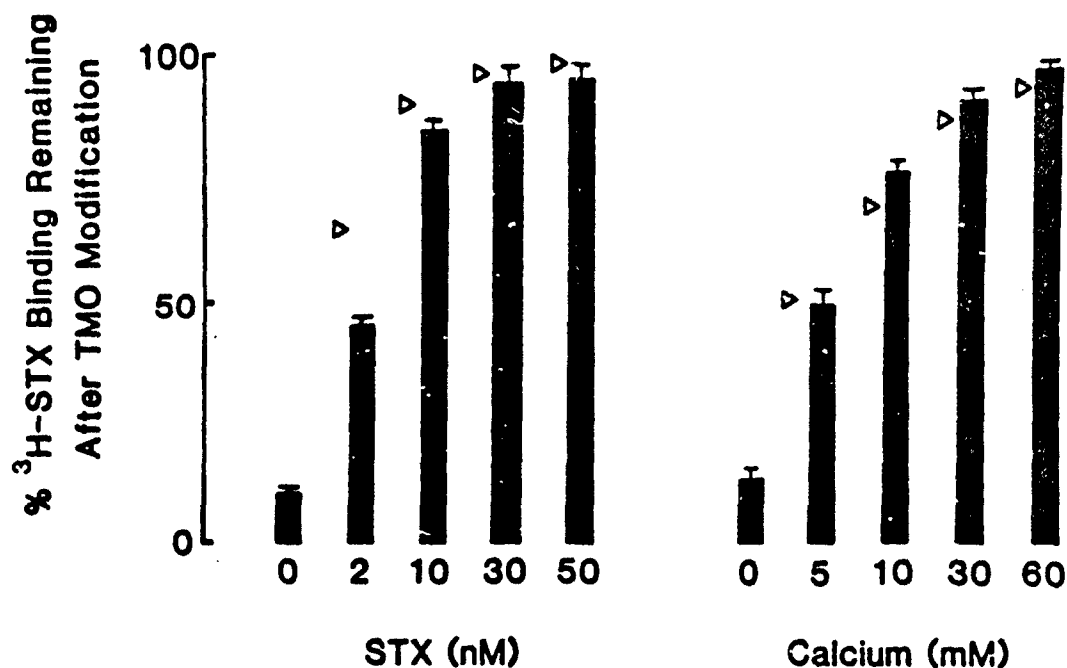


Figure 8. STX and Calcium protection from TMO modification. <sup>3</sup>H-STX binding was measured after the addition of TMO (50-60 nM) in the presence of STX or calcium at the indicated concentration in the reaction mixture. The data are plotted as the mean  $\pm$  standard error of the mean (n=4). Zero STX or calcium represents the modification of the STX binding site by TMO. In some cases TMO modification was variable among different preparations, varying from 10-30% loss of <sup>3</sup>H-STX binding. The arrows indicate the expected protection from TMO modification as described in the text.

ability to protect from chemical modification further supports the hypothesis that calcium and STX can bind to a common site, which is the toxin binding site.

#### TMO modification reduces sodium channel block by extracellular calcium

The following experiments were designed to determine whether the TMO modified site is the same site that binds calcium to reduce the single channel currents. Figure 9 illustrates the current-voltage relationships of a TMO modified sodium channel before and after the addition of 10 mM calcium to the extracellular side. This toxin insensitive sodium channel appears to be nearly insensitive to calcium block following TMO modification. The degree of current reduction is considerably lower than that of the unmodified, STX sensitive sodium channel as was illustrated in Figure 4 and this can be further demonstrated by the following figure.

In Figure 10 the amount of current reduction (fraction blocked) in the presence of calcium is plotted as a function of the applied voltage for both a normal and a TMO modified channel. At all potentials, the TMO modified channel is less sensitive to extracellular calcium than the unmodified sodium channel. The smooth lines were drawn assuming the fraction of the field sensed by the blocking ion was constant ( $D=0.23$ ), while the affinity for calcium at 0 mV ( $K_0$ ) was varied. The line at the top of the figure was calculated using a  $K_0$  of 17.7 mM calcium. The other two lines were determined as the  $K_0$  is increased to 133 and 240 mM calcium. This implies that alterations in the functional groups of the sodium channel, following TMO modification, are responsible for drastically lowering the affinity for calcium. It is not possible, at this time, to determine whether calcium block of TMO modified sodium channels is voltage-dependent.

In recent years there has been considerable interest in the relationship between the structure and the function of ion channels. This study has used chemical modification of single sodium channels in planar lipid bilayers to perturb channel structure and monitor the consequences by recording the channel's behavior before and after chemical modification. The aim was to determine the role(s) played by TMO modified groups on the functional properties of toxin block and ion permeation of sodium channels. This report endeavors to investigate a possible link between ion permeation and toxin binding. Therefore, the following discussion will center on evidence that suggests that a collection of protein residues (or side chains) on the sodium channel, referred to as the toxin binding site, can also bind cations, and that binding to this site is a necessary step in the ion permeation process.

#### Discussion.

When added to the extracellular surface of a sodium channel from rat brain which has been incorporated into a planar lipid bilayer, TMO eliminated STX and TTX sensitivity.  $^3\text{H}$ -STX binding to the rat brain membrane vesicle preparation ( $\text{P}_3$ ) was also eliminated. Furthermore, single channel currents were reduced by 37% following TMO modification. TMO is a very highly reactive carboxyl modification reagent which esterifies carboxyl groups, producing a methyl ester. This produces a dramatic change in the character of the protein residue. A normally negatively charged, hydrophilic group is modified to a neutral, less hydrophilic residue. Therefore, the reduced single channel conductance could be due to a combination of electrostatic and steric factors acting on a permeating ion. This

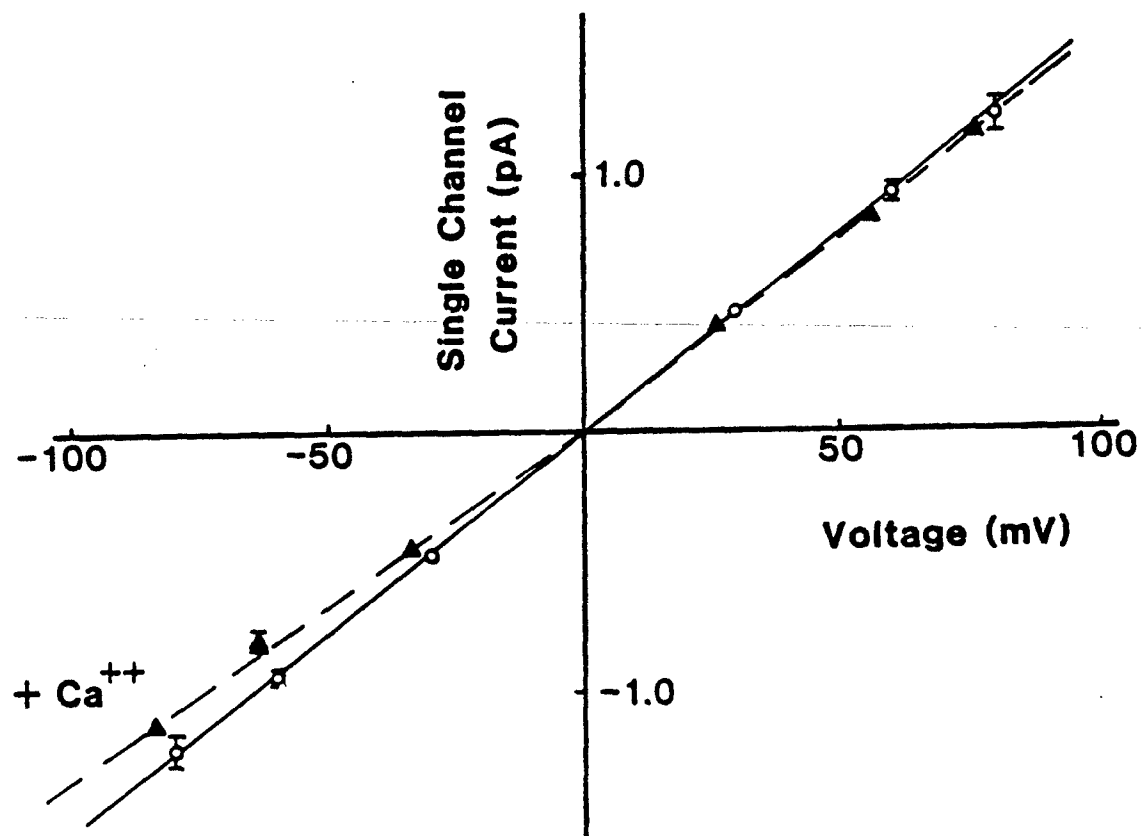


Figure 9. The influence of calcium on TMO modified channels. The current-voltage relationships were obtained from sodium channels incorporated into neutral bilayers bathed by symmetrical 125 mM sodium. Single channel currents were measured following modification by TMO (open circles,  $n=9$ ; see Figure 3) and finally 10 mM calcium was added to the extracellular side. STX (5 nM) added after TMO modification did not block the channels. Each point on the dashed line (closed triangles) represents the single channel current (mean  $\pm$  SEM) at the indicated potential in 1-5 membranes containing TMO modified sodium channels in the presence of 10 mM calcium. The data point at -80 mV was obtained from a single membrane. These STX insensitive channels are nearly insensitive to calcium.

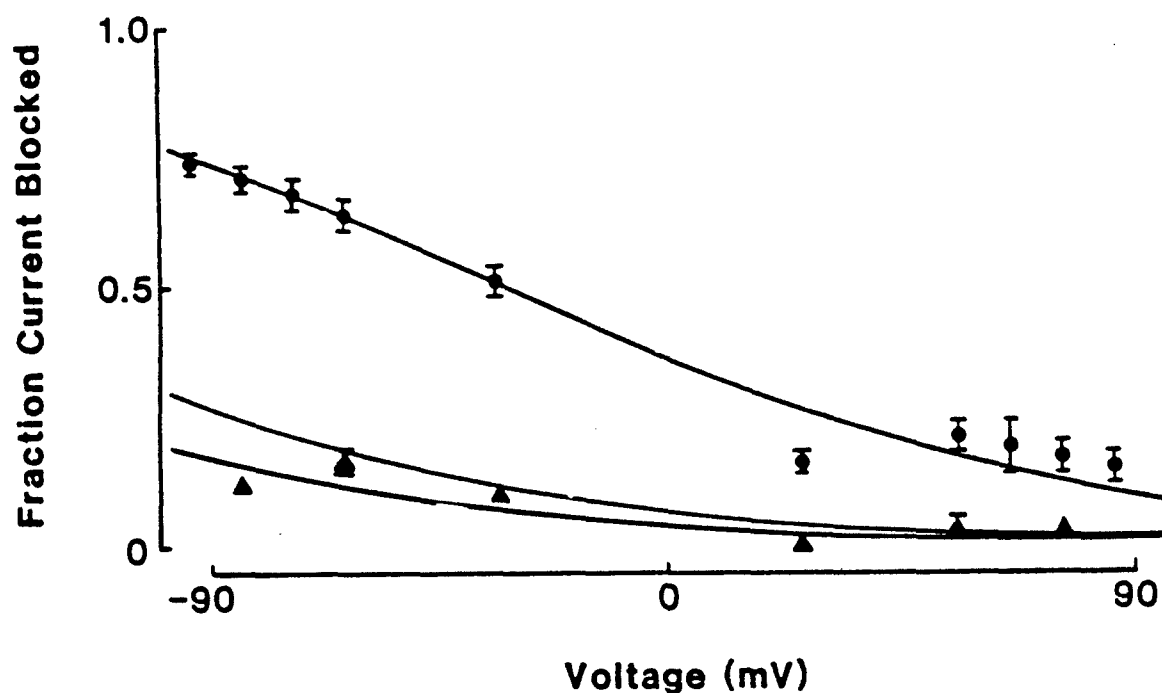


Figure 10. Fraction of current blocked by calcium. The fraction of current blocked by the presence of 10 mM extracellular calcium of single channel currents of normal, STX sensitive sodium channels and TMO modified sodium channels. The fraction of current blocked by calcium was determined from the following equation:  $F_b = (1 - I_m / I_{max})$  where  $I_m$  and  $I_{max}$  are the magnitude of the single channel currents in the presence and absence of 10 mM calcium, respectively, and were obtained from the data in Figures 5 and 9. The smooth curves were calculated as described in the text from equation 2 with the following parameters:  $D$  is 0.23 and  $K_0$  was 17.7, 132.9, and 240 mM calcium (from top to bottom line, respectively).

raises the following questions: 1) Does TMO modification alter the affinity for sodium? or 2) Does chemical modification change the maximum rate of sodium ion passage through the channel? If one assumes that the maximum transport rate of ions through the channel is unaltered, the affinity for sodium has been reduced about 3.3 fold (see next section). On the other hand, if the affinity for sodium is not altered due to TMO modification, then the modified residue(s) would cause the free energy required for sodium ion passage across the membrane to be increased by about 1.5, thus lowering the overall ion permeation rate. The next section will investigate these possibilities in further detail. Due to the simultaneous removal of toxin sensitivity and reduced single channel conductance, the results in this section demonstrate a link between the ion permeation process and the toxin binding and blocking site.

#### Extracellular calcium and single sodium channel currents

When calcium was added to the extracellular surface of a sodium channel incorporated into a planar lipid bilayer, the single channel current was reduced in a voltage-dependent manner. These results suggest that calcium ions may bind to a site on the sodium channel, which is either located approximately 23% of the electrical distance from the extracellular surface or whose affinity for calcium may be indirectly influenced by changes in transmembrane voltage. These results are in agreement with previous work on macroscopic sodium currents in frog myelinated nerve (10) and on single channel current in cultured neuroblastoma cells (18). The following section will present a more detailed and systematic investigation of the dependence of single sodium channel current on sodium and divalent cation concentrations.

#### Calcium and STX: A Coordination site?

It has been suggested that STX and TTX act by plugging the sodium channel, with the guanidinium group entering the mouth of the channel, preventing ions to pass. Since guanidinium permeates the sodium channel, this model appears attractive and links toxin block with ion permeation. In this section, calcium was found to compete for STX block of sodium channels incorporated into a bilayer as illustrated in Table 2. The on (blocking) rate for STX was found to be decreased in the presence of calcium, as would be predicted from simple competition. However, apparent competitive interactions can be more complex and it may be that calcium introduces an unfavorable steric or charge factor, perhaps at some secondary location, which would reduce STX binding. Although the exact origin of the calcium effect is unknown, the apparent  $K_d$  for STX block was increased about 7 fold in the presence of calcium, as determined from both steady state and kinetic analysis. A 5-6 fold increase was found for  $^3H$ -STX binding under similar conditions. In addition, as will be demonstrated in the following section, sodium also competes with calcium to relieve block of single channel current. These results suggest that calcium and sodium competitively inhibit STX binding and block, perhaps by interacting at the same site.

TMO modification caused the sodium channel to become insensitive to toxin blockade and reduced the single channel conductance. Prevention of chemical modification by STX, suggests that the chemically modified site is the toxin binding or blocking site. In fact, the ability of STX to protect against TMO modification can be directly related to its intrinsic affinity for that site as shown in Figure 8. The concentration of STX required to prevent half of the STX

binding sites from being modified was about 3 nM, which is higher than the  $K_d$  for  $^3H$ -STX binding (about 1 nM). Presumably, this may reflect the amount of time the modification reagent is active. In the presence of 1 nM STX, on the average, half of the sites would be occupied by STX. However, since TMO is active for longer than the mean bound time ( $1/\text{dissociation rate}$ ), STX will dissociate from some of the blocked sites, thereby rendering more of them accessible to chemical modification. A similar concentration-dependent protection of the STX binding site was observed when calcium, instead of STX, was present (Figure 8). The estimated  $K_i$  for calcium blockade of single channel currents was found to be between 2-9  $\mu M$  (Table 3); 5  $\mu M$  calcium was able to prevent half the sites from being modified by TMO. Calcium, a low affinity blocker, binds and unbinds at a very fast rate so that the  $K_i$  for current reduction by calcium reflects the average time a sodium channel is occupied by the divalent cation. Due to the nature of calcium block, there may be, in fact, less than 50% of the sites available for modification at the  $K_i$  for calcium block. Although experiments were performed under very different conditions there is close agreement between the sensitivity of calcium block of single channel currents and calcium protection from TMO modification of the  $^3H$ -STX binding site by calcium binding.

Although an apparent complete loss of toxin binding and block is observed after chemical modification, some reduction in current is seen in the presence of calcium. Therefore, toxin binding appears to be lost but the effects of calcium may still be present. This could be attributed to differences between the nature of a high affinity and low affinity site. Toxin binding is extraordinarily sensitive to alterations in STX and TTX structure, indicating that the conformation of most regions of these molecules are important for their action. This suggests that there is a complex interaction between the toxin molecule and its receptor. Therefore, chemical modification of one or more of these groups (sites) on the channel protein, or on the toxin molecules, would have drastic effects on the affinity for toxins. However, complicated interactions would not be expected for calcium, a point charge which would interact with a more restricted region of the protein. Therefore, if TMO is responsible for decreasing the affinity of these sites for calcium, then adding large amounts of calcium or STX may produce alterations in the single channel currents expected for very low affinity blockers.

TMO modification was also found to drastically reduce calcium block of sodium ion permeation through sodium channels (Figure 9). Under identical ionic conditions, calcium blocked unmodified single sodium channel currents in a voltage-dependent manner, but had very little influence on a TMO modified channel. At present two possibilities exist: 1) the affinity for the calcium binding site (or toxin binding site) is drastically reduced (from 18  $\mu M$  to over 200  $\mu M$  at 0 mV) or 2) the voltage-dependent behavior of the site is altered by TMO modification. By increasing the extracellular calcium concentration to 50-100  $\mu M$ , one would be able to construct a current-voltage relationship to investigate whether the divalent cation induced reduction in single channel current of a TMO modified channel is voltage-dependent or voltage-independent. It is concluded that calcium binds to a site located on the extracellular surface, which is a site intimately involved in toxin binding and block. This site, or region of the channel protein, can also bind sodium and is a necessary step in the movement or passage of sodium ions through the sodium channel pore.

**F. Block of Sodium Channels by Calcium** - Effects of divalent cations and membrane surface charge on ion permeation through sodium channels from rat brain.

Ion transport across biological membranes may be influenced by an electrostatic potential difference at the interface between the membrane and aqueous solution. These surface potentials may result from fixed charges located on proteins which are either closely associated with, or imbedded into the cell membrane, or from the presence of membrane lipids with cationic or anionic head groups. These fixed charges would tend to deplete ions of the same charge and concentrate counterions of opposite charge near the membrane surface. In plasma membranes of many mammalian cells, phosphatidylserine (PS) is the predominant negatively charged lipid. The purpose of this study is to investigate the role of membrane surface charge and divalent cations on the movement of sodium through open sodium channels.

BTX-activated sodium channels from rat brain were incorporated into pre-formed bilayers. Both PE and 70% PS/30% PE membranes were employed and channel incorporation occurred readily in either type of bilayer. The surface charge density was measured for both neutral and negatively charged membranes: PE membranes possess little, if any, membrane surface charge (about 0.06 charges/headgroup) while 70% PS membranes have a high surface charge density (about 0.6 charges/headgroup).

**1. Variation of single channel conductance with sodium concentration**

The conductance of the sodium channel was determined from the size of unitary current fluctuations in the presence of varying concentrations of sodium. Single sodium channels were incorporated into a preformed bilayer composed of PE, a neutral phospholipid. Figure 11 illustrates the variation of single channel current as the sodium ion concentration on both sides of the bilayer membrane was altered. In the presence of 1.0 M sodium on both sides of the membrane and less than 100 nM divalents, the single channel current was about 1.9 pA at -60 mV. When the sodium ion concentration was reduced to 0.5 M, the single channel current was about 1.8 pA. As sodium was further reduced, to 0.025 M, the single channel current decreased to about 0.7 pA. This membrane contained two sodium channels and a small amount of STX (1 nM) present on the extracellular side to induce discrete single channel current fluctuations to the closed or blocked state. This maneuver will be used throughout this chapter as a tool to accurately determine the magnitude of the single channel current.

With asymmetrical sodium, the current-voltage relationship was linear over the entire voltage range studied ( $\pm 90$  mV) as shown in Figure 12. These channels are ohmic, displaying no current rectification, indicating symmetry of the sodium channel protein with respect to ion permeation. The single channel conductance for a sodium channel incorporated into a neutral bilayer bathed by symmetric 500 mM sodium is 28.9 pS while, it is 12.7 pS when the sodium ion concentration is decreased to 25 mM. In both cases, sodium was the only monovalent cation present, with less than 100 nM divalent cations.

Figure 13A illustrates the variation of the single sodium channel conductance in neutral and negatively charged membranes as the sodium ion concentration on both sides of the bilayer membrane was altered. In neutral membranes (PE), the single channel conductance displays simple saturation behavior des-



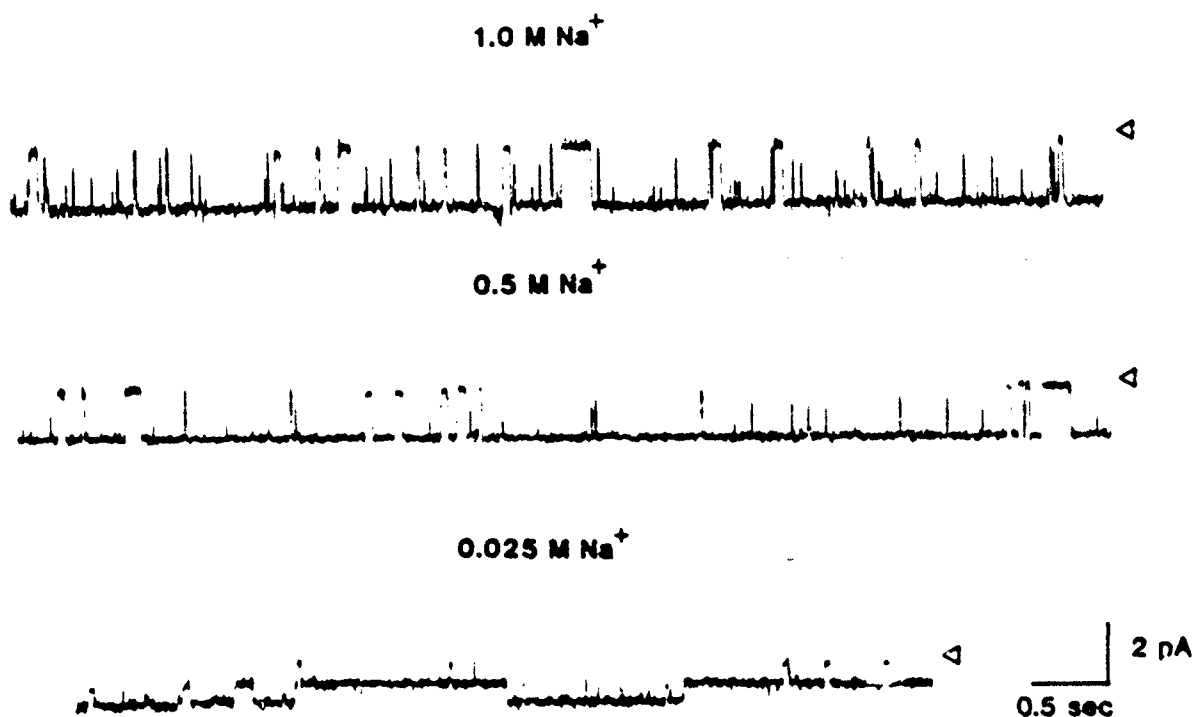


Figure 11. Single BTX-activated sodium channel current fluctuations. Sodium channels from rat brain were incorporated into neutral (PE) membranes. The solutions contained asymmetrical 1.0 M, or 0.5 M, or 0.025 M NaCl and less than 100 nM divalent cations. The potential in each experiment was held at -60 mV. In these records upward current fluctuations represent channel closings and the zero current levels are indicated by the arrows. The membrane at the bottom of the figure contains two sodium channels and a small amount of STX is present on the extracellular side. These records were filtered at 150 Hz.

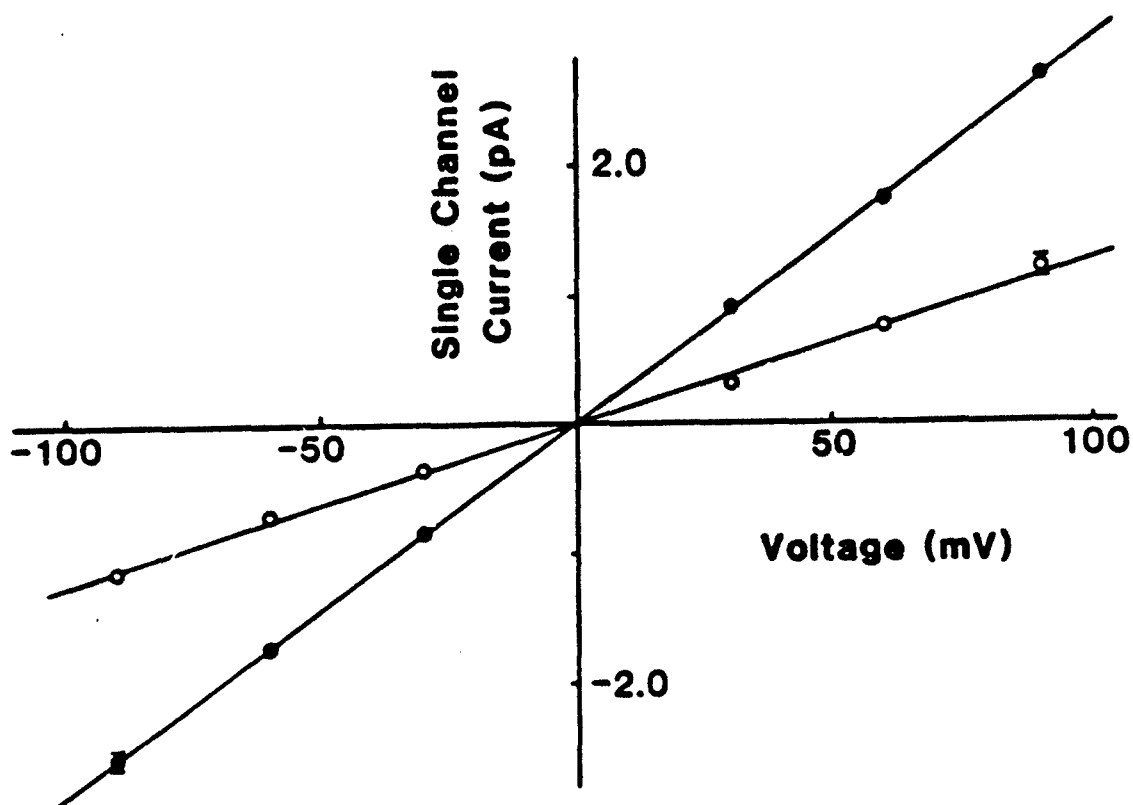


Figure 12. Open channel current-voltage relationship in neutral membranes. Sodium channels were incorporated into neutral membranes bathed by asymmetrical 25 mM NaCl (open circles) or 500 mM NaCl (closed circles) and less than 100 mM divalent cations. Single channel currents were measured at the indicated potentials. The data points represent mean  $\pm$  SEM from 7 to 16 membranes. The single channel conductances calculated from these linear relationships were  $12.7 \pm 0.2$  pS for 25 mM sodium and  $28.9 \pm 0.7$  pS in the presence of 500 mM sodium.

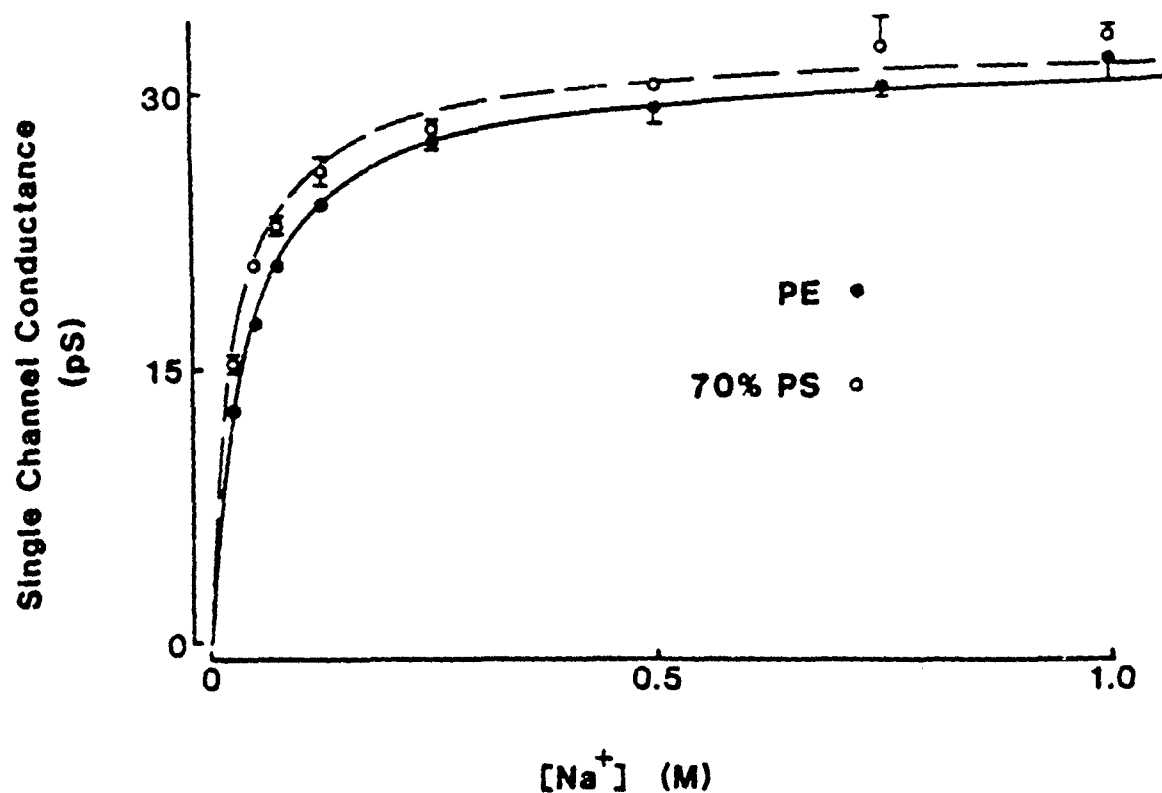


Figure 13A. Single channel conductance as a function of the sodium ion concentration. Single channel conductance-concentration relationship for neutral (closed circles) and negatively charged (open circles) membranes. Each point represents the slope of linear current voltage relationships, as illustrated in Figure 12, for sodium channels at the indicated sodium concentration on both sides of the membrane (less than 100 mM divalent cations). In neutral and negatively charged membranes the conductance can be described by a rectangular hyperbola. The data at each point was determined from 3 to 30 membranes.

cribed by the following relationship:

$$G = G_{\max} \cdot [Na] / ([Na] + K_m) \quad \dots (3)$$

where  $G$  is the measured single channel conductance,  $G_{\max}$  is the maximum conductance, and  $K_m$  is the apparent dissociation constant for sodium. Sodium channels incorporated into negatively charged membranes (70xPS) display a similar saturation behavior which is also well described by a rectangular hyperbola (equation 3). In negatively charged (70x PS) membranes, the open single channel current was also ohmic over the voltage range studied. The Scatchard plots of these results illustrated in Figure 13B show that this simple one ion, one site relationship holds over the entire sodium concentration range studied and that a rectangular hyperbola accurately describes the variation in single channel conductance with symmetric sodium for single sodium channels in neutral and negatively charged membranes. In both membrane types, the single channel conductance appears to saturate between 31 and 32 pS in the presence of less than 100 nM divalent cations. From close examination of the Scatchard plots, the single channel conductance appears not to completely saturate but continues to increase, suggesting either that more than one ion can occupy the channel only under extreme ionic conditions. In addition to saturation of the single channel conductance, a small shift in half maximal conductance was observed as the bilayer membrane was altered. In neutral membranes, the  $K_m$  for sodium is 37 nM, while in negatively charged bilayers the  $K_m$  is 28 nM sodium.

## 2. Block by different divalent cations in asymmetrical sodium

When divalent cations are present in the intracellular and extracellular solutions, a sodium channel becomes asymmetric in its ability to pass inward and outward current. Figure 14A illustrates the effects of addition of three different divalent cations on single sodium channel currents with symmetrical 125 nM sodium. Divalent cations were added to both sides of single sodium channels incorporated into bilayer membranes composed of neutral phospholipids (PE) to minimize alterations in membrane surface charge. Single channel current records were collected in the presence of low amounts of STX (1-3 nM) to induce discrete current fluctuations. Quite independently of the type of divalent cation present, there was a greater reduction of the single channel current at negative potentials (inward sodium current) than at positive potentials (outward sodium current). Manganese was the most potent inhibitor of both the inward and outward movement of sodium through sodium channels. Magnesium was only slightly less potent than calcium; both divalents reduced the inward and outward current components.

The current-voltage relationship for each divalent cation is shown in Figure 14B. In the presence of divalent cations, there is a characteristic asymmetric current-voltage relationship. The sodium channel appears to rectify, passing outward current far more easily than inward current. Block of inward current is dependent on membrane voltage, in that as one hyperpolarizes the membrane from -30 mV to -90 mV, the amount of current reduction is increased about 1.5 fold. A similar voltage-dependent block of inward currents was reported in the previous section when only extracellular calcium was present. Also each of these divalent cations induced a voltage-independent reduction of outward currents in which the current-voltage relationship for positive potentials is linear. For calcium and strontium, the outward current is linear when measured up to 140 mV indicating no influence of voltage on the potency of

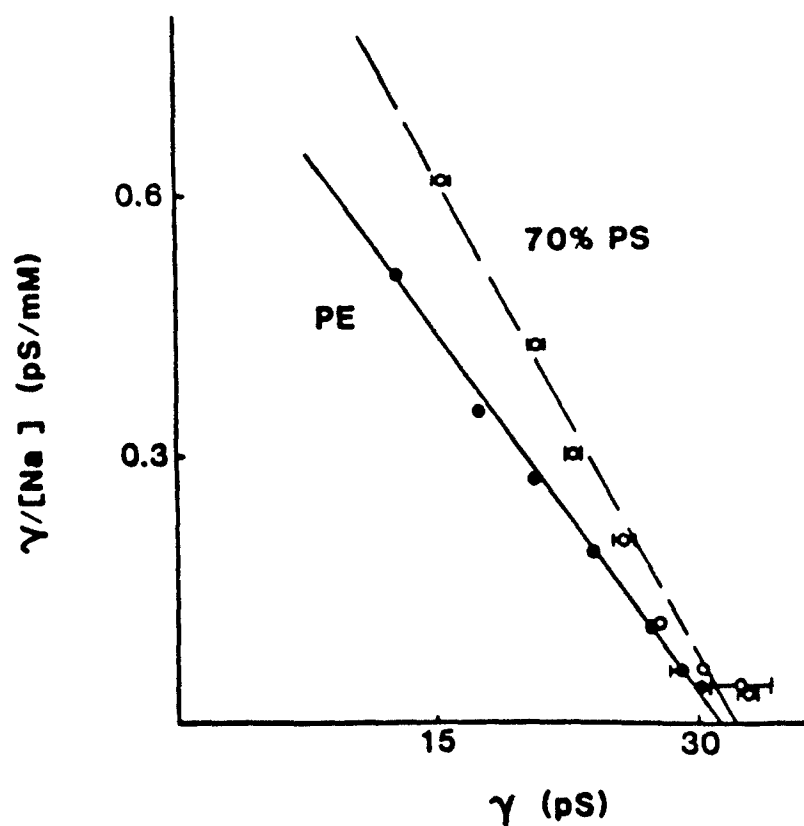


Figure 13B. Scatchard plots of the same data, indicating the convergence of both sets of data to the maximum conductance of 31-32 pS and a deviation in the slope indicating a change in the apparent dissociation constant (correlation coefficient was 0.98 for each ion).

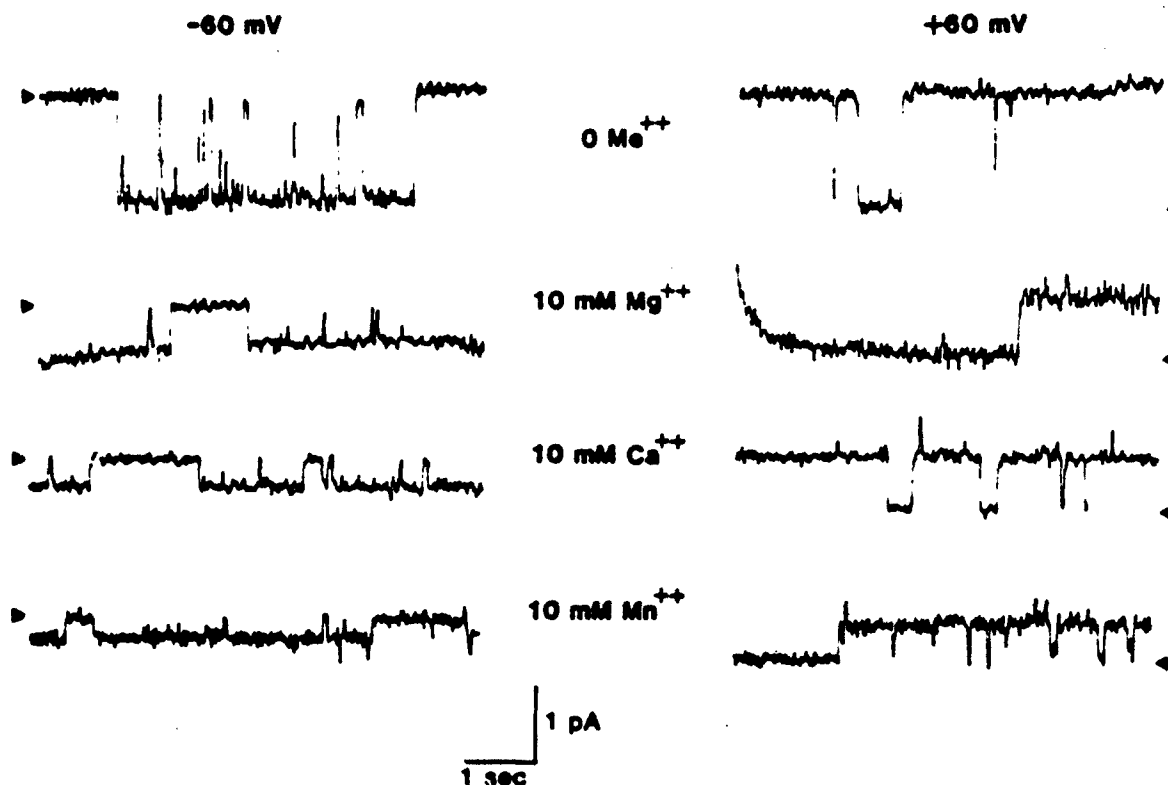


Figure 14A. The effects of different divalent cations on single sodium channel currents. Current fluctuations were measured at 60 and -60 mV. Each membrane contained a single sodium channel incorporated into neutral (PE) membranes with 125 mM NaCl on both sides of the membrane and a low concentration of STX (1-5 nM) added to the extracellular side. Under these conditions, single channel currents were reduced in the presence of 10 mM magnesium, calcium, or manganese on both sides of the membrane. The closed, or STX blocked, non-conducting state, is indicated by the arrows. In the magnesium records the slow drift in the current is due to the decay of the capacitive current following the voltage clamp pulse.

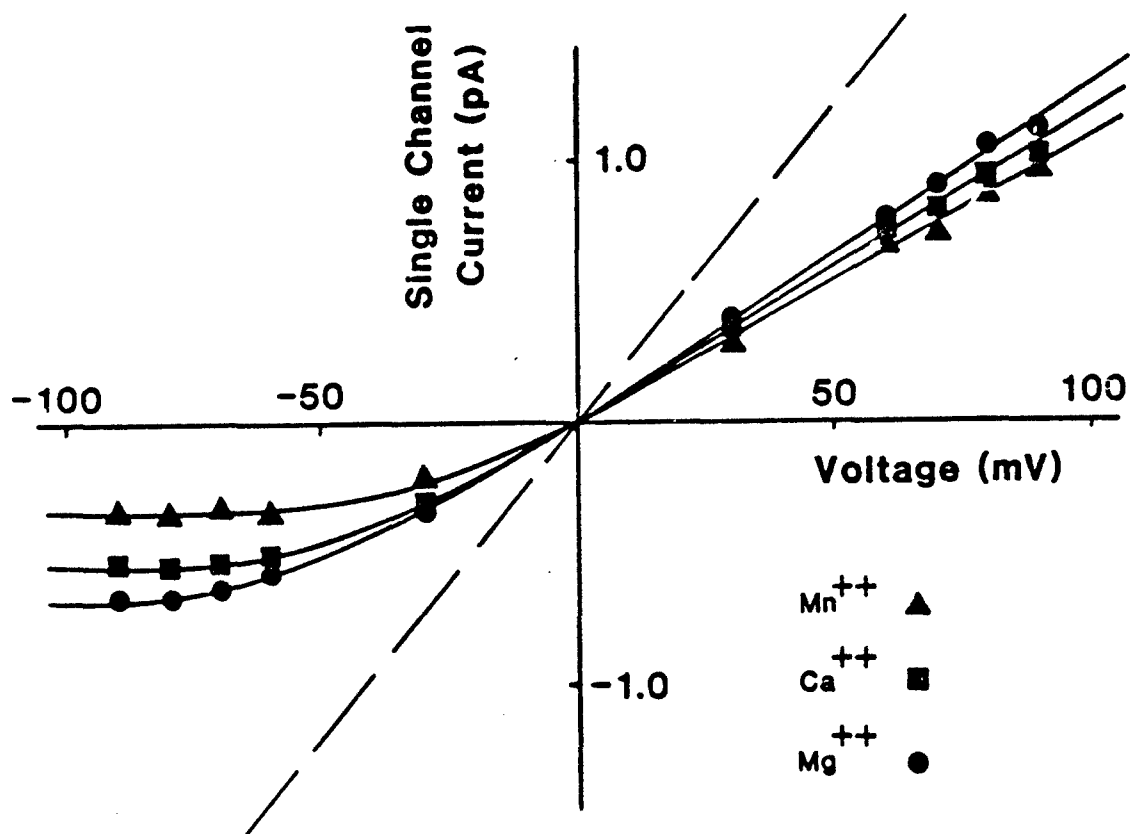


Figure 14B. Open channel current-voltage relationships for single channel membranes such as in 14A. The dashed line represents the control in which the channels were bathed by 125 mM sodium solution and less than 100 mM divalent cations. When 10 mM magnesium (closed triangles), or calcium (closed boxes) or manganese (closed circles) was added to both sides of the membrane, the single channel currents were reduced. The smooth curves were drawn by eye.

current block.

Both voltage-dependent block of inward current and voltage-independent block of outward current may be seen more clearly in Figure 14C, where the fraction of current blocked ( $F_b$ ) by divalent cations as determined from the following relationship was plotted as a function of voltage:

$$F_b = 1 - (I_m / I_{max}) \quad \dots (4)$$

where  $I_m$  is the single channel current measured from discrete current steps in the presence of the divalent cation, and  $I_{max}$  is the single channel current step prior to exposure to divalents. Both components of block were observed for manganese, magnesium, and calcium as shown in Figure 14C. At positive potentials, the fraction of outward current reduced by the divalent cation is constant, i.e., independent of the applied voltage. The horizontal lines drawn between the data points on Figure 14C represent the mean fraction of outward current blocked by divalents at positive potentials. At negative voltages, there is an obvious influence of the transmembrane potential on the amount of divalent cation induced reduction of single inward sodium channel current. The smooth curves at negative potentials were determined from the following relationship:

$$F_b = 1 / 1 + (K_0 / [Me^{2+}]) \cdot \exp(zDFE/RT) \quad \dots (5)$$

where  $F_b$  was determined from equation 4,  $K_0$  is the concentration of the divalent cation ( $Me^{2+}$ ) which gives half maximal block,  $z$  is the valence of the blocker molecule,  $D$  is the fraction of the membrane field sensed by the blocker,  $E$  is the applied voltage, and  $R$ ,  $T$ , and  $F$  have their usual meanings. The data at negative potentials follow a Boltzmann distribution (equation 5) and fail to describe the voltage-independent block of outward current by each divalent cation at positive potentials. In addition, the voltage-independent (horizontal relation) block at positive potentials can not describe the voltage-dependent block at negative potentials.

Table 3 shows the relative potencies of current block by different divalent cations for the voltage-independent and voltage-dependent components. For each divalent cation tested the  $K_i$  for block was estimated from the following relationship:

$$K_i = (1 - F_b^{-1}) \cdot [Me^{2+}] \quad \dots (6)$$

where  $F_b$  is obtained from equation 4 and  $[Me^{2+}]$  is the divalent cation concentration. For the voltage-independent outward current block, the average single channel current reduction obtained between 30 and 90 mV was used to determine the apparent inhibition constant for each divalent cation listed in Table 3. For voltage-dependent block of inward currents at negative potentials for each divalent cation the values in Table 3 were obtained from the non-linear least squares fit of equation 6 to the data. Note that qualitatively similar voltage dependence is observed for block of inward current by each divalent cation except for barium (Table 3). This inconsistency appears to reside in the lack of fit of a Boltzmann distribution (equation 5), primarily due to the data point at -30 mV. When this point is omitted,  $D$  becomes approximately 12% and is close to the value obtained from other divalents. Furthermore, the order of potency of the divalent cations for the block of inward current is the same for block of



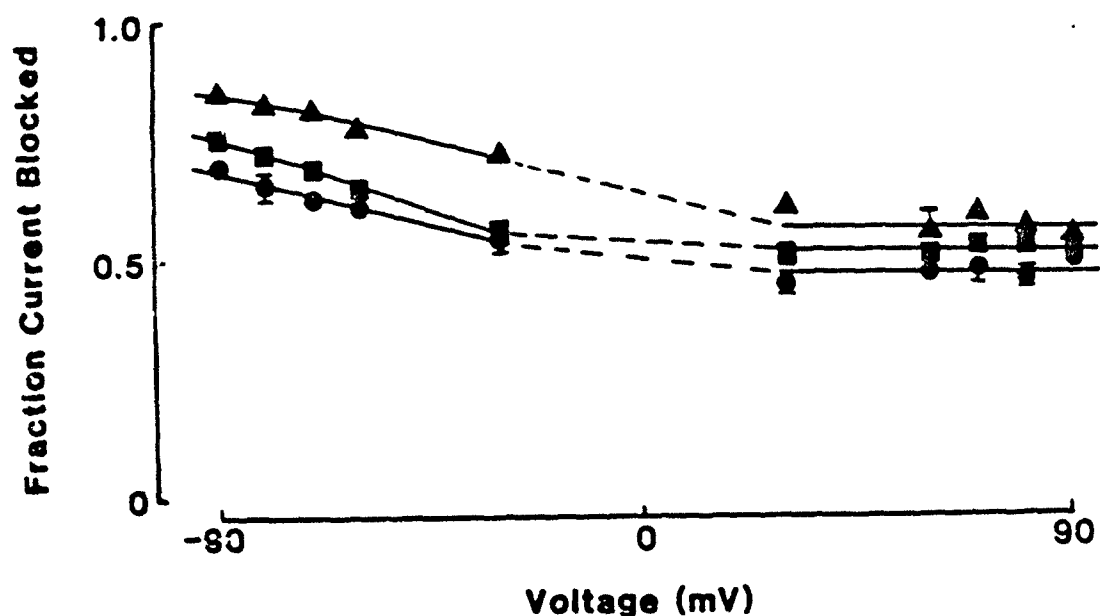


Figure 14C. Fraction of current blocked vs. applied potential for the data in B. The horizontal lines represent the average fraction of current blocked at positive voltages and corresponds to the voltage independent component of calcium induced outward current block. The smooth curves were calculated from non-linear least squares fit of a Boltzmann distribution (equation 5) to the data at negative voltages with the following parameters: Magnesium,  $K_0$  is 11.65 mM and  $D$  is 0.13; Calcium,  $K_0$  is 13.0 mM and  $D$  is 0.19 and; Manganese,  $K_0$  is 6.2 mM and  $D$  is 0.17. The dashed lines were drawn to connect the voltage-dependent and voltage-independent components of divalent cation block, and indicates the range of potentials where the block was not evaluated.

outward current. These results suggest a direct, specific interaction of divalent cations for a site, or collection of sites, closely associated with sodium ion permeation through sodium channels which has the following affinity sequence:  $Mn^{2+} > Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$ .

Table 3. Effect of divalent cations on single channel currents.

Divalent cation	$K_i$	Inward current block	
	outward current block	$K_i (-80mV)$	$D(x)$
Manganese	7.9 mM	1.2 mM	16.6
Calcium	9.6 mM	2.1 mM	18.8
Magnesium	11.7 mM	2.8 mM	12.6
Strontium	23.8 mM	11.8 mM	17.4
Barium	30.9 mM	19.8 mM	6.5

All data points were obtained from 2-6 membranes. The  $K_i$  for outward current block was obtained from the mean fractional block of outward current at positive potentials and equation 6. The parameters for inward current block were first obtained from a non-linear least squares fit of a Boltzmann distribution (equation 5) to the fractional block of inward current (negative potentials). Then the  $K_i$  was determined from equation 6. Each data point, for the respective divalent cation, was within 0.002 and 0.02 (RMS standard deviation) from the best fit line.

### 3. Effect of divalent cations under asymmetric sodium conditions

When the sodium concentration was identical on both sides of the membrane the sodium channel current-voltage relationship was ohmic (Figure 12). However, the sodium channel displays a small degree of apparent current rectification under more physiological ionic conditions as shown in Figure 15. Sodium channels were incorporated into neutral (PE) membranes with 125 mM NaCl, 5 mM KCl on the extracellular side and 5 mM NaCl, 125 mM KCl on the intracellular side and the divalent cation concentration on both sides was below 100 nM. The slope of the current-voltage relationship at potentials more negative than -60 mV indicates a single channel conductance for inward current of about 19 pS. With symmetric sodium (125 mM) with no potassium present, the single channel conductance was 24 pS. When 2 or 10 mM calcium was added to the extracellular side, the inward single channel current was reduced. The influence of transmembrane voltage on reduction of inward sodium ion movement is nearly identical to that found for symmetric sodium conditions. For 10 mM calcium there is about a 48% reduction in inward current at -30 mV and 74% reduction at -90 mV. From equation 5, the reduction of outward currents is equivalent to calcium sensing about 27% of the transmembrane field ( $K_0 = 17.8$  mM calcium). This indicates that under different ionic conditions, asymmetric vs symmetric sodium, the characteristic voltage dependence of calcium block of inward single channel current is similar and is in close agreement with that found by other investigators (10, 18). In addition, physiological concentrations of extracellular calcium (2 mM) can reduce the single channel current in a voltage-dependent manner as illustrated in Figure 15. The inward current is reduced by about 20% at -40 mV and by 44% at -90 mV. Therefore, near the normal resting and threshold potentials, under "physiological" ionic conditions, the single sodium channel current is significantly reduced by extracellular divalent cations.

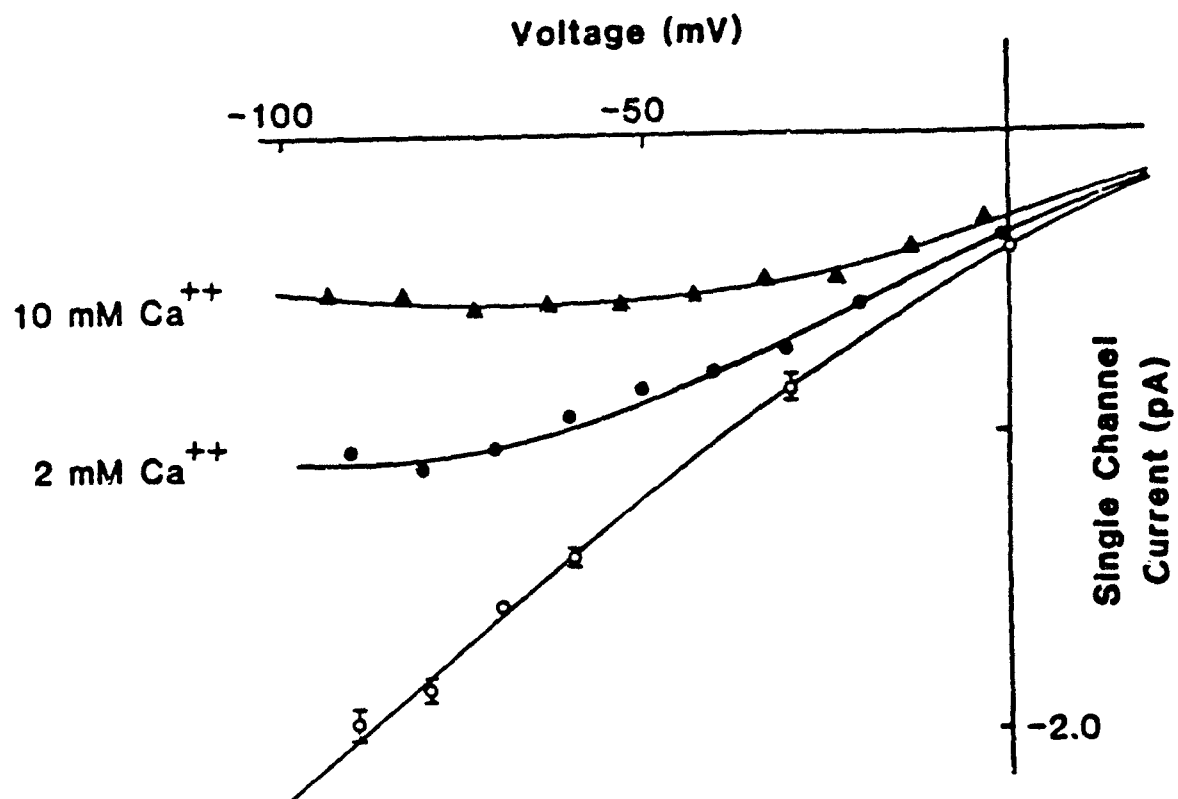


Figure 15. Single channel currents under stylized "physiological" conditions. Open single channel current-voltage relationships for sodium channels incorporated into neutral membranes with 125 mM sodium, 5 mM potassium on the extracellular side and 5 mM sodium, 125 mM potassium on the intracellular side and less than 100 nM calcium (open circles;  $n=9$ ). Under these stylized cellular ionic conditions, reduction of the single channel current is observed after the addition of 2 mM (closed circles) or 10 mM (closed triangles) calcium to the extracellular side. Each point represents the means  $\pm$  SEM from 2-4 membranes. The smooth curves were drawn by eye.

#### 4. Effect of asymmetric calcium on gating.

Besides reducing the single channel current, extracellular divalent cations have been shown to influence the kinetic behavior of the open and closed states of the sodium channel (19). Figure 16 shows the effects of altering the divalent cation concentration on the extracellular side of the sodium channel. Sodium channels were incorporated into neutral (PE) membranes with 250 mM sodium on both sides. When 10 mM calcium was added only to the extracellular side, the single channel current was reduced (42%) and the channel spent more of its time in the closed state. As the extracellular calcium was doubled the single channel current is further reduced (59%), the channels spent an increasing amount of time in the closed state and the shift of the activation curve to more depolarized potentials becomes more dramatic. This is consistent with the results by other investigators in that extracellular divalents produced shifts in the curve that relates sodium channel activation to membrane voltage. When divalents were added to the intracellular side, at a concentration to equal that on the extracellular side, the reduction in current remained (about 59%) but the apparent shift in the activation curve was eliminated, presumably by removing asymmetries in membrane surface charge produced by asymmetric divalents. The amount of current reduction in the presence of 20 mM extracellular calcium was almost identical to the current block when the calcium concentration was 20 mM on both sides of the bilayer. These results indicate that there are charged groups on the intracellular surface of the channel which may influence the gating machinery.

#### 5. Effect of calcium on single channel current under normally saturating sodium conditions.

In addition to the influence of voltage, the block of the single channel current by divalent cations is concentration-dependent. Figure 17A shows current fluctuations in the presence of the indicated concentration of calcium added to both sides of a single sodium channel incorporated into a neutral membrane with symmetrical 250 mM sodium. As the concentration of calcium is increased, the amount of current reduction or blockade of inward and outward current is increased. As shown in Figure 14, for each calcium concentration, there is a greater reduction of single channel current at negative potentials than at positive potentials.

The current-voltage relationship for each calcium concentration is shown in Figure 17B. The influence of voltage on divalent cation block of both inward and outward current appears to be independent of the calcium concentration. At each calcium concentration, the single channel current displays rectification; outward current is greater than inward current under symmetrical conditions.

Separation of voltage-dependent block of inward current and voltage-independent block of outward current can be more clearly illustrated in Figure 17C, where the fraction of current blocked (determined from equation 4) is plotted as a function of voltage. As the concentration of calcium is increased, the single channel current is reduced and the influence of voltage remains unaltered. The lines in the figure were determined from equation 5 as described for Figure 14C. At positive potentials, the horizontal lines are calculated from the mean current block for the voltage-independent component. The smooth curves at negative potentials were obtained from a Boltzmann distribution (equation 5)

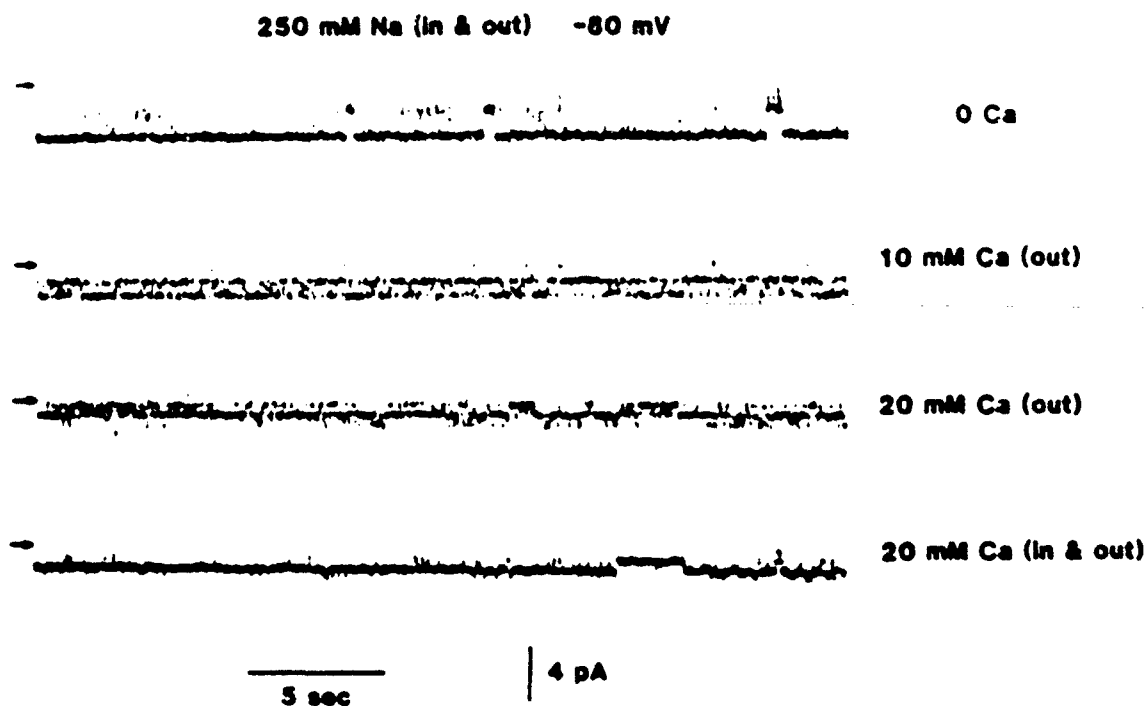


Figure 16. Influence of calcium on sodium channel gating. Two sodium channels were incorporated into neutral (PE) membranes bathed by symmetrical 250 mM sodium. At -80 mV the channel was open for more than 85% of the time (in the presence of BTX). When 10 mM calcium is added to the extracellular side the single channel currents are reduced and the channels spend an increasing amount of time in the closed state. Increasing calcium to 20 mM further reduces single channel currents and increases the time the channel spends in the closed state. When the calcium concentration is equalized by the addition of 20 mM calcium to the intracellular side, the single channel current reduction remains unaltered, however the channel is nearly always open as prior to calcium addition.

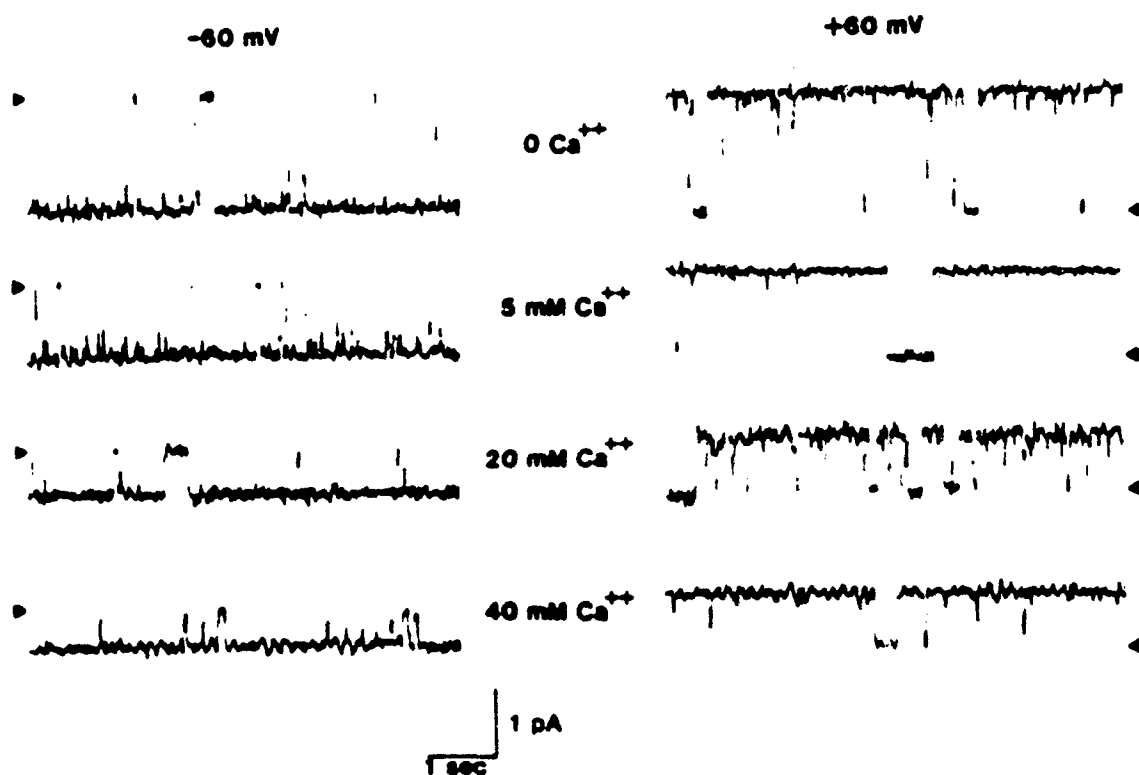


Figure 17A. Single sodium channel current fluctuations in the presence of increasing concentrations of calcium on both sides of the bilayer. Single sodium channels were incorporated into neutral (PE) bilayers with symmetrical 250 mM sodium and a low concentration of STX (2-5 nM) on the extracellular side. The current records shown were taken at 60 and -60 mV; the closed states are indicated by the arrows.

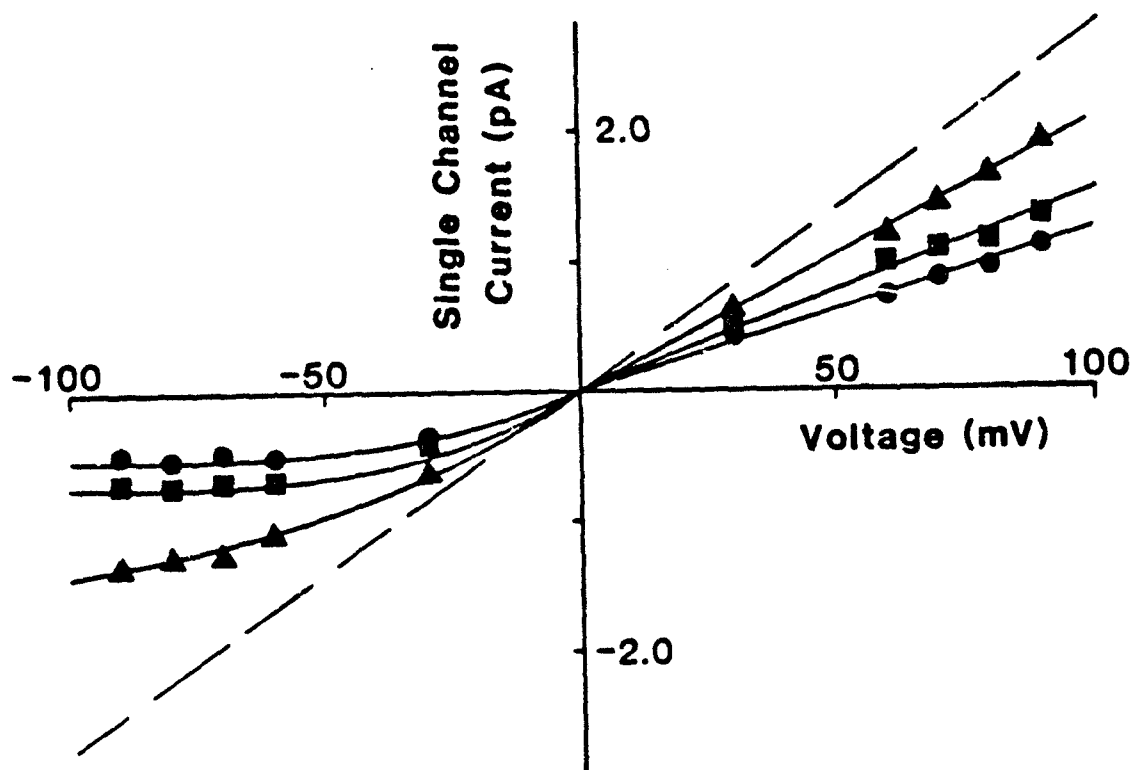


Figure 17B. Open channel current-voltage relationship for sodium channels under conditions identical to those in A in the absence of divalents (dashed line) or in the presence of 5 mM calcium (closed triangles), 20 mM calcium (closed boxes), or 40 mM calcium (closed circles) on both sides of the membrane. Each point represents the means  $\pm$  SEM obtained from 2-8 membranes. The smooth curves were drawn by eye.

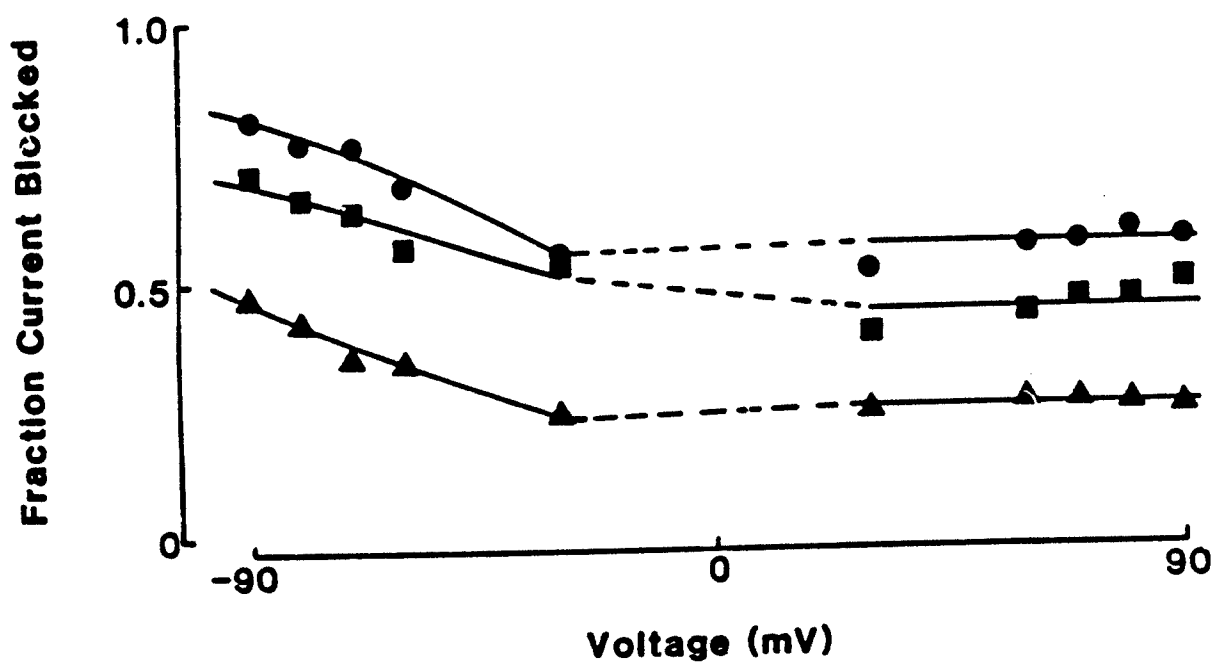


Figure 17C. Fraction of current blocked vs. potential. The data were obtained from B and the lines were drawn as described for Figure 14. The parameters for the Boltzmann distribution are as follows: For 40 mM calcium the  $K_0$  is 62.7 mM and D is 0.27; for 20 mM calcium the  $K_0$  is 27.9 mM and D is 0.16 and; for 5 mM calcium the  $K_0$  is 27.8 mM and D is 0.22. As described before, the dashed line indicates the range of potentials where Fb was not evaluated.



and represents the voltage-dependent component of divalent cation block of sodium ion movement through single sodium channels.

Figure 18 illustrates the saturating behavior of divalent cation block. Figure 18A is a plot of the fraction of current blocked as a function of the calcium concentration (asymmetric) at 90 and -90 mV. Note the saturating behavior of both inward and outward current block. The smooth curves were drawn from the following relationship:

$$F_b = (F_{b_{max}} \cdot [Ca^{2+}]) / ([Ca^{2+}] + K_i) \quad \dots (7)$$

where  $F_b$  is the fraction of current blocked obtained from equation 4,  $F_{b_{max}}$  is the theoretical maximum blockable current,  $[Ca^{2+}]$  is the free calcium concentration on both sides of the incorporated sodium channel, and  $K_i$  is the apparent inhibition constant for calcium block. From the double reciprocal plots in Figure 18B, divalent cation block appears to saturate for both the voltage-independent (90 mV) and voltage-dependent (-90 mV) components. Furthermore, because of the close fit of equation 7, the data suggest that one calcium ion interacts with each sodium channel to block the movement of sodium through its pore. The X-intercept or apparent calcium affinity for current block is different for each potential. The  $K_i$  for calcium is 4.4 mM at -90 mV and 9.8 mM at 90 mV. In addition, the Y-intercept or  $F_{b_{max}}$  changes with potential (see below).

Table 4 lists the  $K_i$  and  $F_{b_{max}}$  for calcium block at each potential, determined from Scatchard plots of the data in Figure 18. The applied voltage appears to reduce the apparent  $K_i$  for calcium for inward current (4.4-10.3 mM) and has a smaller influence on calcium affinity of outward current reduction (6.4-9.8 mM). In fact, the effect of voltage on the calcium affinity for reduction of the outward current is negligible, if one omits the 90 mV determination (6.4-7.4 mM). Although the current-voltage relationship for positive potentials provides no indication of non-linearity, i.e., block of outward current appears to be voltage-independent, there appears to be a small influence of voltage on the  $K_i$  and  $F_{b_{max}}$ , the parameters determined from data transformations. By close inspection of these parameters at 90 mV, not only does the  $K_i$  increase but the  $F_{b_{max}}$  increases, suggesting a possible interaction between the two parameters which, at present, cannot be explained. For inward current block by calcium, the apparent voltage dependence was determined from the following equation which is derived from the Boltzmann distribution (equation 5):

$$K_i(E) = K_0 \exp(zDFE/RT) \quad \dots (8)$$

where  $E$  is the applied potential,  $z$  is the valence of the blocking ion,  $D$  is the fraction of voltage sensed by the blocking ion,  $K_0$  is the apparent affinity constant for the divalent cation blocker at 0 mV, and  $R$ ,  $T$ , and  $F$  have their usual meanings (10). The distribution of the apparent inhibition constant ( $K_i$ ) for calcium at negative voltages (i.e., inward current block) indicates that the site of calcium action is located about 28% of the electric field from the extracellular side. In addition,  $F_{b_{max}}$  is influenced by the applied voltage. While positive voltages appear to have a small influence on the maximum current reduction by calcium, at negative voltages the maximum  $F_b$  approaches one. Qualitatively similar results were observed by strontium block of inward and outward currents (data not shown). However, the  $K_i$  for strontium (10.9 mM at -60 mV) is higher than calcium, reflecting its lower affinity.

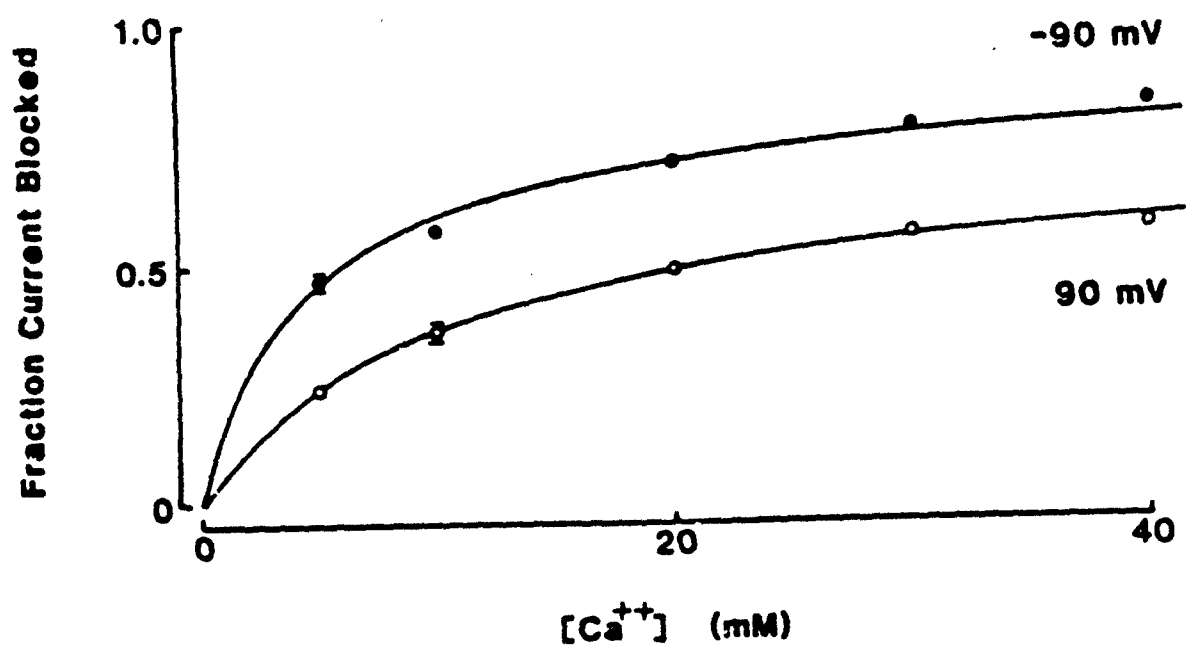


Figure 18A. Saturation of current block by asymmetrical calcium in the presence of 250 mM sodium. Plot of the fraction of current blocked at 90 and -90 mV at the indicated calcium concentrations. The data were obtained from current-voltage relationships like those of figure 17B. The curves were calculated as described in the text, and the parameters were obtained from Table 4.

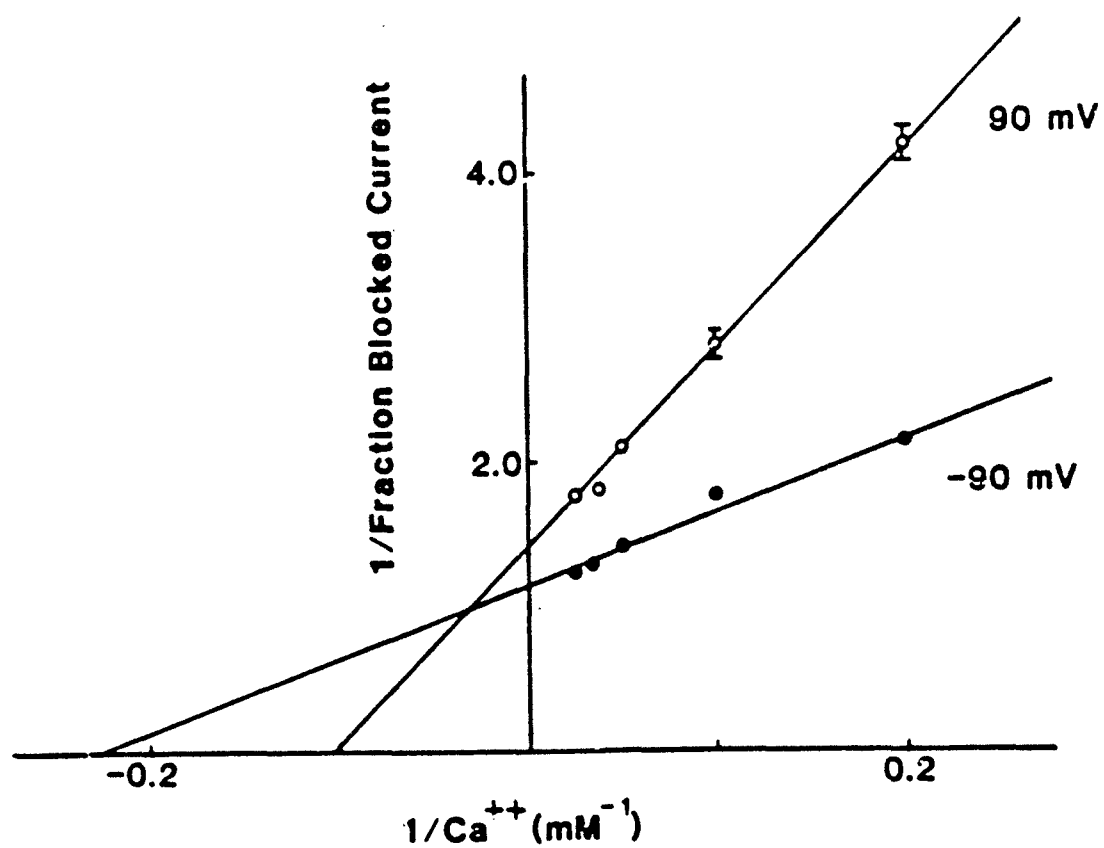


Figure 18B. Double reciprocal plot of the data in A. Note the changes in the apparent inhibition constant as shown by the difference in the x-intercept.

Table 4.  $K_i$  and  $Fb_{max}$  for calcium block with symmetrical 250 mM sodium.

Voltage (mV)	$K_i$ (mM)	$Fb_{max}$	Voltage (mV)	$K_i$ (mM)	$Fb_{max}$
-30	10.3	0.76	30	6.4	0.55
-60	6.7	0.78	60	6.6	0.59
-70	7.2	0.86	70	7.3	0.63
-80	4.6	0.81	80	7.4	0.62
-90	4.4	0.80	90	9.8	0.71

Values were obtained from Scatchard analysis of data from those of Figure 18A. The correlation coefficient for the linear regression lines ranged between 0.90 to 0.99 and each point represents data collected from 3-6 membranes.

#### 6. Effects of calcium at normally non-saturating sodium concentrations.

Similar effects of calcium are observed at sodium concentrations where the single channel conductance is not saturated. Figure 19 is a plot of fractional blocked current ( $Fb$ ) vs symmetric calcium addition at 90 and -90 mV, under the same conditions as Figures 17 and 18, except that the sodium concentration has been reduced to 75 mM. The smooth curves were drawn from equation 16. Under these conditions, the reduction in single channel current displays saturating behavior similar to that observed with symmetrical 250 mM sodium. Similar changes in the  $K_i$  for calcium and  $Fb_{max}$  with applied potential were observed, however, the apparent inhibition constant for calcium block is higher than that at 250 mM sodium.  $Fb_{max}$  at negative potentials (inward current block) varied between 0.71 at -30 mV to 0.85 at -90 mV, while at positive potentials (outward current block) it was between 0.60-0.69. The apparent inhibition constant ( $K_i$ ) for calcium for block of inward current was between 1.2 mM at -30 mV and 0.47 mM at -90 mV. For outward current block, the  $K_i$  was between 1.2 and 1.6 mM calcium. Under identical conditions, with the addition of strontium as the divalent cation blocker, similar alterations in the  $K_i$  for strontium and  $Fb_{max}$  were observed with voltage. However, the absolute values of the  $K_i$  for strontium (2.7 mM at -60 mV) are higher due to strontium's lower blocking affinity. Furthermore, the  $K_i$  for strontium at 250 mM sodium is higher than at 75 mM sodium. These results suggest competition between the divalent blocker and sodium (the permeating ion).

#### 7. Competition between calcium and sodium.

The following illustrates competition between the divalent cation and the permeating ion. Figure 19 shows the fraction of current blocked by symmetric addition of 10 mM calcium, as the sodium ion concentration on both sides of a neutral (PE) membrane is altered. As the sodium ion concentration is decreased from 750 to 75 mM, the fraction of current blocked becomes greater. However, the voltage-dependent and voltage-independent components of block are still evident. The lines in Figure 20 were calculated as described above for Figures 14C and 17C: at negative potentials the smooth curve represents the non-linear least squares fit of a Boltzmann distribution to the fraction of inward current blocked by calcium, while at positive potentials, the block of outward current is independent of voltage.

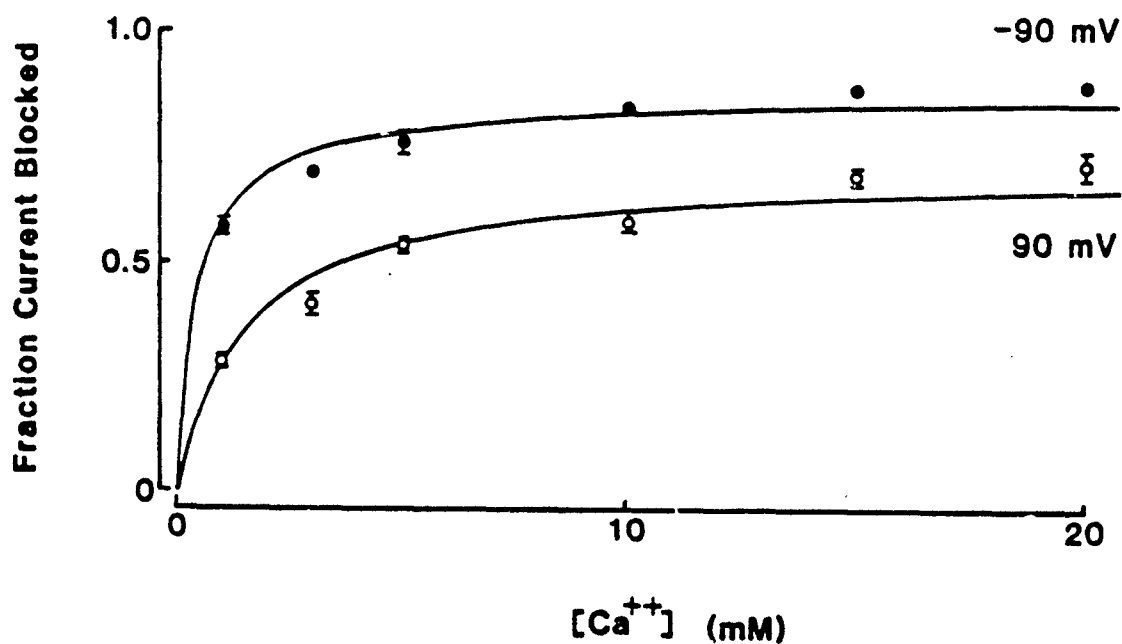


Figure 19. Saturation of fraction of current blocked by calcium in the presence of 75 mM sodium. Data was obtained as in Figures 17 and 18 in which the fraction of current blocked was evaluated at the indicated calcium concentrations on both sides of the membrane at 90 and -90 mV. Each point represents the means  $\pm$  SEM from 2-5 membranes. The curves were determined as described in the text with the following parameters: at -90 mV  $K_1$  is 0.47 mM calcium and  $F_{bmax}$  is 0.84; at 90 mV  $K_1$  is 1.47 mM calcium and  $F_{bmax}$  is 0.7. Note the similarity to that of Figure 18A.

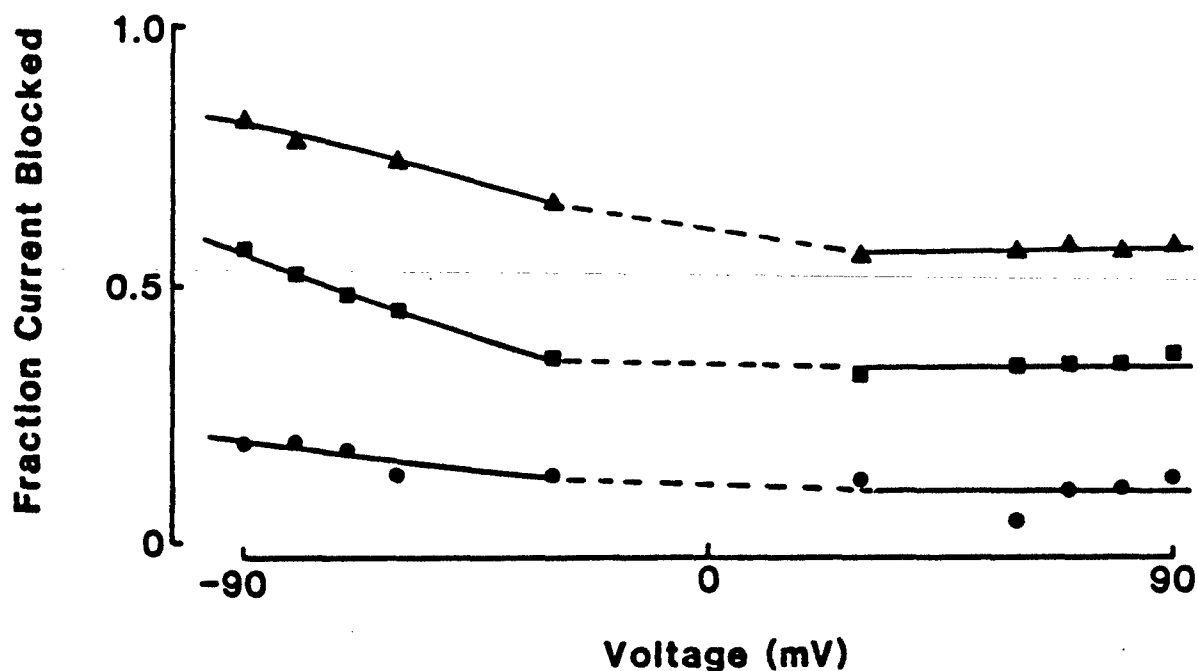


Figure 20. Competition between calcium and sodium. Fraction of current blocked vs. applied potential for symmetric 75, 250, and 750 mM sodium in the presence of 10 mM calcium on both sides of the membrane. Sodium channels were incorporated into neutral membranes and each point represents the means  $\pm$  SEM for 3-8 membranes. The lines in the figure were determined as in Figures 14 and 17. The parameters for the Boltzmann distribution are as follows: For 75 mM sodium the  $K_0$  is 7.7 mM for calcium and  $D$  is 0.17; for 250 mM sodium the  $K_0$  calcium is 28.6 mM calcium and  $D$  is 0.18 and; for 750 mM sodium the  $K_0$  is 93.0 mM calcium and  $D$  is 0.12.

When sodium channels are incorporated into negatively charged membranes, nearly identical results are observed as illustrated in Figure 21. The fraction of current blocked at -30 mV is plotted as a function of the sodium ion concentration on both sides of the membrane for both neutral and negative membranes. Even at low ionic conditions, where the effects on ion permeation of calcium induced alterations in membrane surface charge would be more prominent, no difference can be observed. Upon evaluating these results at all potentials, there was no significant difference of calcium block of sodium channels incorporated into negative or neutral membranes. However, at a few potentials, a small (5-10%) difference was observed and occurred only at high sodium concentrations (above 500 mM), similar to those observed in Figure 21. Therefore, it appears that membrane lipid surface charge plays little, if any, role in the block of sodium channels by divalent cations.

The results in Figure 20 and 21 suggest competition between the divalent cation and sodium. Figure 22A illustrates the variation in single channel current as the sodium ion concentration is altered in the absence and presence of calcium. In the absence of divalents, the single channel current (at -60 mV) is described by a rectangular hyperbola as was shown in Figure 13A. In the presence of 10 mM calcium, the single channel current increases as the sodium ion concentration is increased, approaching that of controls, where calcium is absent. The smooth curve was calculated from a variation of equation 3:

$$I = I_{\max} \cdot [Na^+] / ([Na^+] + K_m).$$

These results suggest that calcium competes with sodium in reducing inward single channel currents.

The double reciprocal plot of single channel current vs sodium in Figure 22B reveals a large change in the apparent  $K_m$  for sodium in the presence of calcium, while only a small change in the maximum conductance was observed. In the presence of calcium, the apparent  $K_m$  for sodium is increased from 37 mM to about 500 mM. Similar results were obtained for all applied potentials. The apparent dissociation constant for sodium varied from 150 to 250 mM at positive potentials, where outward current block is independent of voltage. For the voltage-dependent component, the apparent dissociation constant for sodium increased from 300 mM at -30 mV to about 1.2 M at -90 mV. In addition, strontium displays similar competitive interactions with sodium. At -60 mV, the apparent  $K_m$  for sodium is increased to about 300 mM in the presence of 20 mM strontium. From enzyme kinetics, a competitive inhibitor is defined as a substance (e.g., calcium) which combines with a receptor (sodium channel) preventing substrate (e.g., sodium) binding, so that the binding of the substrate and inhibitor is mutually exclusive. In this example, sodium binding to the sodium channel results in current flow and calcium inhibits the movement of sodium through sodium channels. With this scheme, the sodium channel's affinity for sodium ions will be reduced in the presence of the inhibitor while the maximum current will be unaltered. Although the  $K_m$  for sodium is drastically increased, the apparent maximum current is also increased as indicated by a change in the y-intercept in Figure 22B in the presence of 10 mM calcium. At more negative potentials, the theoretically determined maximal current is increased by about 2. Therefore, the exact type of interaction between sodium may be more complicated than simple competition (see below).

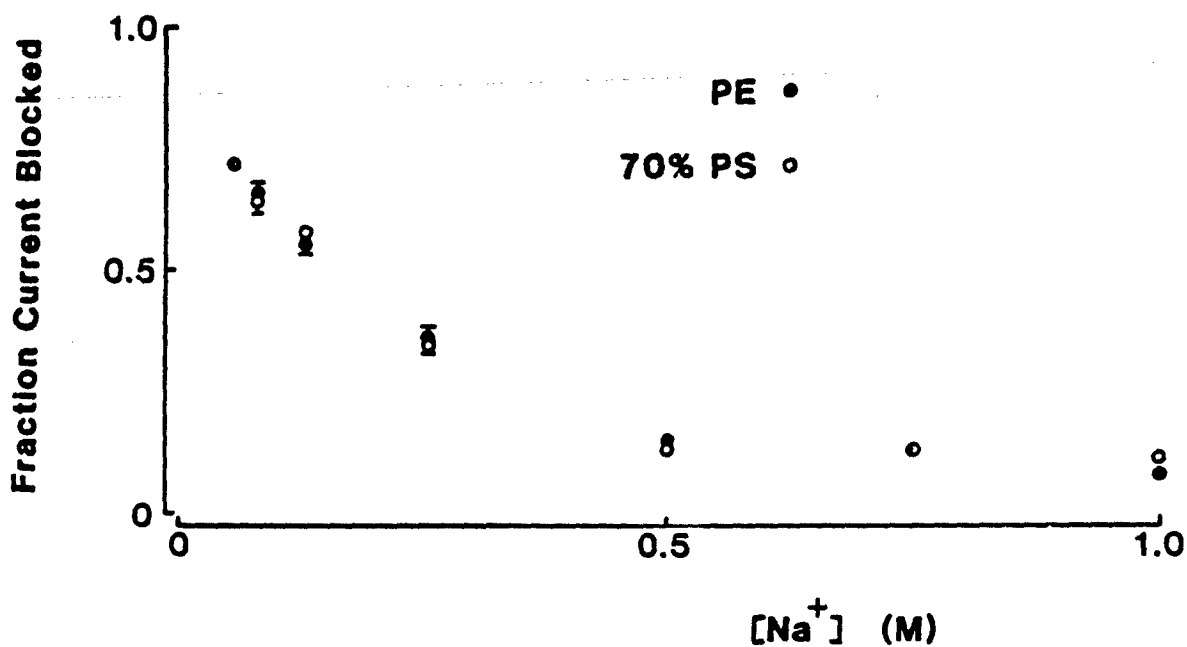


Figure 21. Lack of an effect of membrane surface charge on the competition between calcium and sodium. Fraction of current blocked by symmetric 10 mM calcium at the indicated sodium concentrations. The single channel currents were evaluated at -30 mV in neutral (closed circles) and negatively charged (open circles) membranes.



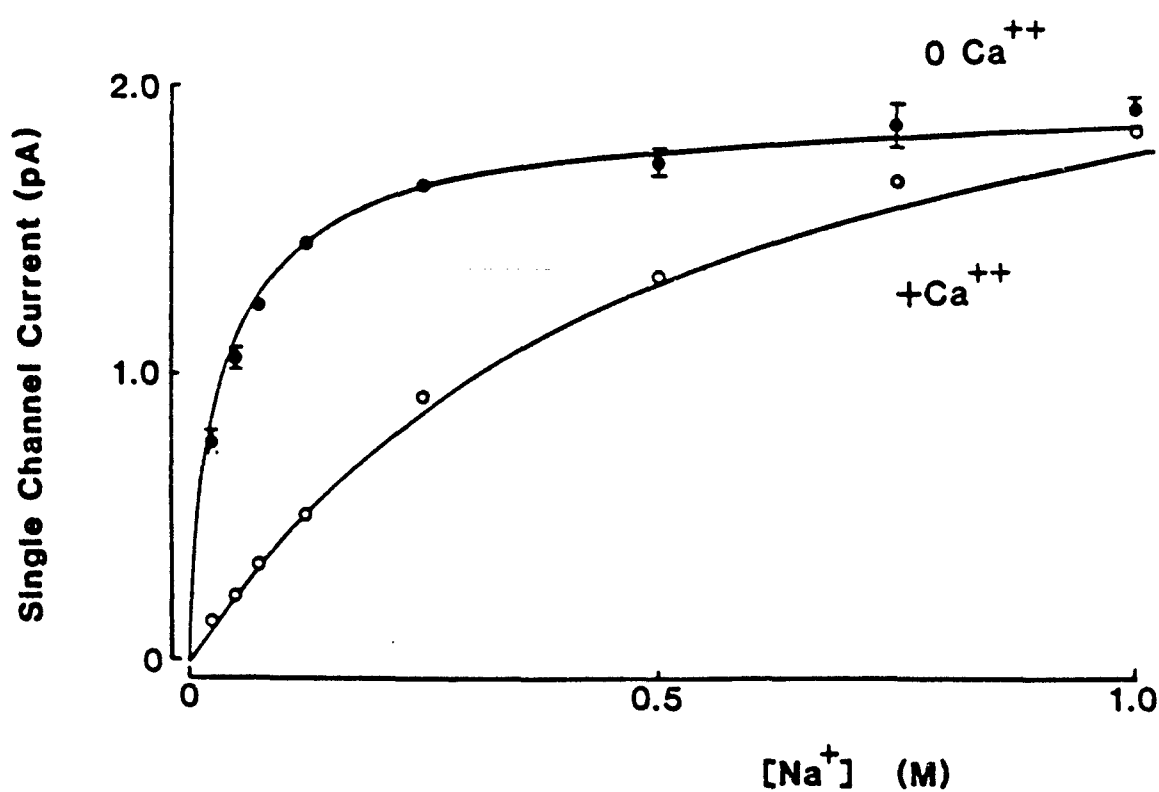


Figure 22A. Competition between sodium and calcium. Single channel current as a function of the sodium ion concentration in the absence (closed circles) and presence (open circles) of 10 mM calcium. Single sodium channel current fluctuations incorporated into neutral membranes were evaluated at -60 mV and were obtained from experiments as in Figure 20 and 21. The curves were determined as described in text.

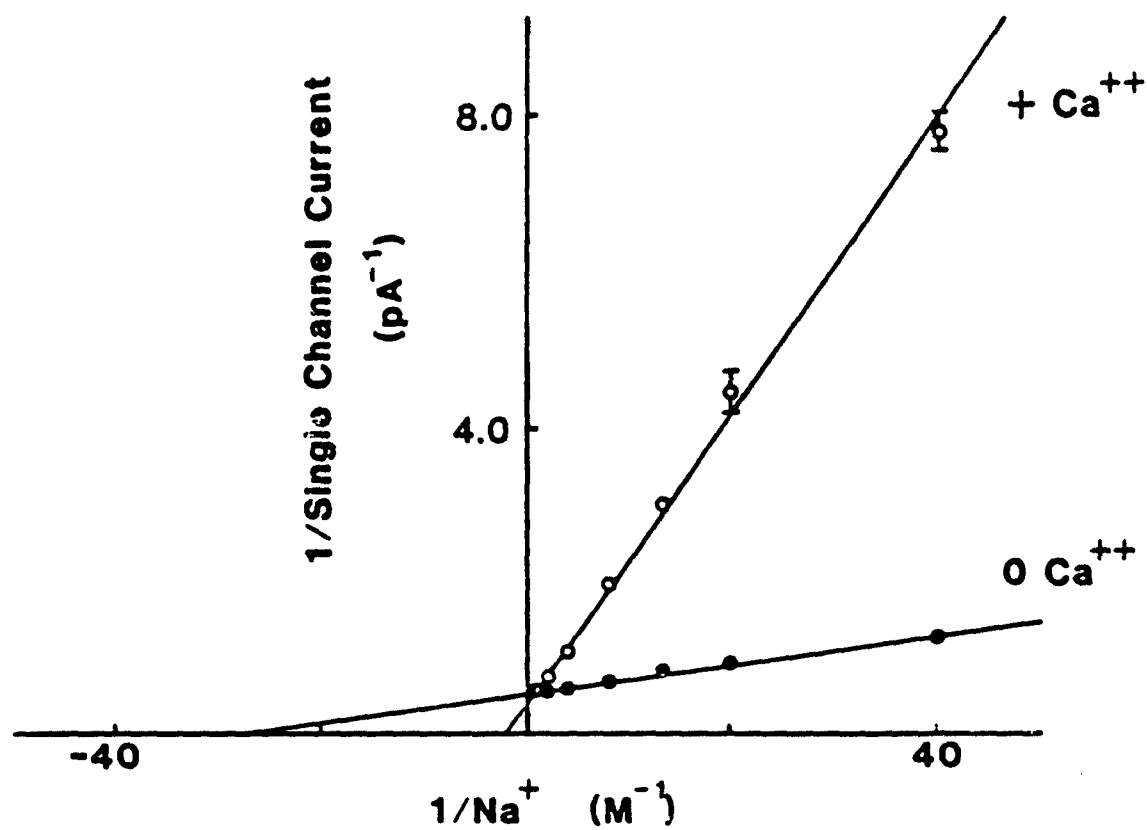


Figure 22B. Double reciprocal plots of the data in A. Note the change in the X-intercept, indicating that the apparent dissociation constant for sodium ( $K_m$ ) in the presence of calcium.

## Discussion

Effects of alterations in the ionic and membrane phospholipid environment on current through single sodium channels have been analyzed to gain information about the structural organization of the sodium channel. The following discussion will focus on the relative effects of membrane surface charge and divalent cations on sodium ion movement through the sodium channel pore.

### 1. Characteristics of the single sodium channel conductance.

The results presented above demonstrate variations in the conductance of single sodium channels in planar lipid bilayers, when exposed to different ionic conditions. In addition to the influence of divalent cations on single channel currents, which will be discussed below, changes in the amount of sodium present at the intracellular and extracellular end of the sodium channel may also influence the movement of sodium through sodium channels. A clear example can be seen when one compares Figure 14 and Figure 15 in the absence of appreciable divalent cations (less than 100 nM). When the sodium ion concentration was symmetrical (125 mM) the single channel conductance was 24 pS. However, under stylized "physiological" conditions where there is a small amount of potassium present on the extracellular side and a large amount on the intracellular side, the single channel conductance was about 19 pS. Even with physiological concentrations of monovalent cations, the single sodium channel conductance in the absence of divalent cations is higher than the 2-15 pS which has been reported for other sodium channels using patch clamp (18,20). The apparent discrepancy is probably due to the presence of divalent cations at the external surface of sodium channels in intact cells. In those reports, the extracellular solutions contained 0.5-50 mM  $\text{Ca}^{2+}$  and/or 1-2.5 mM  $\text{Mg}^{2+}$ , both of which are capable of reducing the single channel current as illustrated in Figure 16. In this study, with cellular ionic conditions, 2 mM calcium added to the extracellular side of sodium channels reduced the single channel current about 30% at -60 mV (Figure 15) (18). By correcting for these alterations in the ionic environment, the values for single channel conductance reported here are close to those reported in the literature for a variety of cell types. However, the degree to which BTX effects the movement of sodium through sodium channels or the block by divalent cations is presently unknown.

### 2. Single channel saturation

Sodium ions permeate through ion channels by a process different from simple diffusion. The sodium channel contains at least one binding site located within the transmembrane field that is accessible from either the internal or external cellular environment. The results presented above demonstrate that the magnitude of current through a single sodium channel is a function of the ionic composition of the solutions which bathe the intracellular and extracellular surfaces. As the sodium ion concentration is increased symmetrically, the single channel conductance increases non-linearly. In fact, the relationship between single channel conductance and sodium saturates as the sodium ion concentration is raised above 200 mM and is described by a rectangular hyperbola. Similar results were obtained with skeletal muscle T-tubule sodium channels (21). Early analysis of voltage-dependent membrane conductances assumed that ion movement through biological membranes is independent of the presence or concentrations of either permeant or impermeant ions (22). However, an obvious deviation from the "independence principle" is demonstrated by the saturation of the single sodium

sodium channel, with its lipid environment, incorporates into the bilayer, some native lipid domain from the brain membrane vesicle remains around the channel, insulating it from the bulk lipid composing the bilayer membrane. At present one cannot exclude this possibility. However, based on the calculations above, the channel protein is large enough to account for the insulation of the ion permeation pathway from the membrane surface environment.

#### 4. Divalent cations and ion permeation.

Block of the ion permeation pathway by divalent cations has been attributed to two main factors: non-specific screening and a direct, more specific interaction between the divalent cation and the sodium channel. Both factors may either separately, or in concert, influence sodium ion movement through sodium channels. The following discussion will focus on the evidence for a direct interaction of divalent cations with the sodium channel.

The characteristic behavior of all divalent cations tested was that the reduction in single channel current consisted of two components, a voltage-dependent block of inward current and a voltage-independent block of outward currents. This is clearly seen in Figures 14C, 17C, and 20. The voltage-dependent component appears to occur by divalents binding to a site located approximately 20-25% of the electrical distance from the extracellular surface. In the previous chapter, 10 mM extracellular calcium reduced the inward current in a voltage-dependent manner. This current reduction is nearly identical to that obtained by symmetrical 10 mM calcium (about 60% at -60 mV). In addition, reduction of outward currents by asymmetric calcium (10 mM) is about 2.6 times greater than that observed by extracellular calcium alone (10 mM) at the same potential with symmetrical 125 mM sodium. Therefore, it appears that the voltage-dependent and voltage-independent components of divalent cation block are distinct.

Many different divalent cations were found to reduce the single channel currents. The order of efficacy was  $Mn^{2+} > Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$ . In this report, block by all divalent cations had similar characteristics and the apparent voltage dependence for the current reduction was similar, suggesting that divalents bind to a common site located on the sodium channel. Barium was the only divalent cation whose ability to reduce single sodium channel currents appeared to be less dependent on voltage. The binding sequence is the same for the voltage-independent block of outward current as well. The sequence for blocker potency is also quite different from that observed for divalent cation binding to phospholipids. For example, phosphatidylserine (PS), which is the most common negatively charged phospholipid found in most mammalian cell membranes has the following divalent cation binding sequence:  $Mn^{2+} > Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+}$ . These results support the notion that the site at which divalent cations interact to reduce single sodium channel currents is not a phospholipid headgroup, but is part of the sodium channel. Furthermore, addition of membrane surface charge or alterations in the permeant ion concentration appeared to have no effect on the characteristics of calcium block of the single sodium channel current. These results suggest that the effects are specific for the channel and not an indirect effect of alterations in membrane surface potentials.

An interesting question arises when evaluating the total fraction of current which can theoretically be blocked by divalent cations. As seen from the saturation curves in Figure 18A and 19 at -90 mV, the maximum blockable current does not approach 100%. The most probable explanation is that calcium ions

channel conductance. This suggests that sodium ions do not move independently through the aqueous pore of the sodium channel as if in solution, but instead interact with at least one site located within the sodium channel pore as they pass through. Furthermore, due to the fit of the data to a rectangular hyperbola, there appears to be a single saturable binding site in which one sodium ion (substrate) can bind to one sodium channel (receptor). Therefore, the channel appears to operate as a single ion channel in which no more than one ion can occupy the pore at one time (23-26) at least within the sodium concentration range studied (0 - 1 M). These non-linearities or interactions are further guides to the microscopic energy wells and peaks within the channel in which a sodium ion must pass as it crosses the membrane and form the basis for an ongoing rate theory analysis of ion permeation through the sodium channel.

### 3. Effect of membrane surface charge.

In this section, the question of the extent to which membrane surface charge affects ion permeation is discussed. Several investigators have reported that sodium channels are relatively insensitive to alterations in membrane surface charge as determined by extracellular divalent cation addition. In this report, the effect of membrane surface charge was determined directly, in the absence of divalents. One might expect that increases in membrane surface charge could cause accumulation of positively charged sodium ions near the mouth of the channel, thereby increasing the local sodium ion concentration (27,28). This, in fact, would explain the small, almost negligible decrease in the apparent dissociation constant for sodium in the presence of negatively charged membranes as observed in Figure 13. Furthermore, alterations in membrane surface charge did not affect calcium induced reductions in single channel currents as illustrated in Figure 20. Even at low ionic strength, where the effects of alterations in membrane surface charge would be expected to be greatest, none were observed. In fact, a minimal effect of membrane surface charge on calcium induced reduction of sodium ion movement was observed only at high sodium concentrations (greater than 500 mM), an unexpected result based on present theories on electrostatic forces or potentials adjacent to membranes. Furthermore, the characteristics of calcium block at 75 and 250 mM symmetrical sodium were nearly identical, suggesting a lack of significant contribution of the membrane lipid environment to the ion entry into the channel, indicating that the mouth of the channel is, to some degree, insulated from the membrane lipid environment.

These results might be expected based on current knowledge of the molecular structure of the sodium channel. Assuming that the sodium channel protein has a molecular weight of 300,000 and is arranged as a cylinder, 80 Angstroms in length, the surface area of the face of the channel exposed to the intracellular and extracellular solutions would be about 80 angstroms in diameter. Levinson and Ellory (29) using irradiation inactivation, determined that the sodium channel behaved as a spherical protein, which spans a lipid bilayer, also about 80 angstroms in diameter. A channel protein of this size would not permit the mouth of the sodium channel or ion permeation pathway to be significantly influenced by the lipid environment or alterations in its surface charge (27). This calculation assumes the surface of the channel is flush with the membrane phospholipid surface. Alternatively, the channel may extend out into the extracellular solution from the plane of the membrane.

An alternative explanation for the inability of added membrane surface charge to influence sodium permeation through sodium channels is that, when a

permeate the sodium channel, thereby carrying some fraction of the total ionic current. However, the maximum fraction of current blocked ( $F_{\text{max}}$ ) by calcium is lower at -30 mV than at -90 mV. It seems unlikely that calcium ions permeate at a higher rate at low potentials than at higher potentials. In addition, attempts to detect divalent cation permeation of sodium channels in the absence of monovalent cations have been unsuccessful. At 250 and 75 mM sodium,  $F_{\text{max}}$  remains constant at positive potentials (about 0.6), where the current reduction is independent of voltage. At negative voltages, which favor the voltage-dependent component,  $F_{\text{max}}$  increases from about 0.6-0.9 as the membrane becomes more hyperpolarized. These observations are inconsistent with calcium permeating the sodium channel and argue against a simple blocking model. These results indicate that the exact nature of divalent cation interaction with the sodium channel may be more complicated than can be described by a simple rectangular hyperbola, even though the data appear to be well fit by this type of analysis.

#### 5. Extracellular calcium and sodium channel gating.

In addition to the reduction in single channel current by divalent cations, extracellular calcium can influence the gating machinery of the sodium channel, causing the channel to be closed at potentials where it would normally be open (Figure 16). In this example, it is clear that the single channel current is reduced by calcium. However, the average current is reduced to a greater extent, due to a decrease in the length of time the channel is open. Thus, extracellular calcium also reduces the macroscopic (averaged) sodium current by reducing the probability of channel opening. Similar effects of extracellular divalents have been reported before (19,30).

#### 6. Competition between sodium and calcium.

If the sodium channel operates as a single ion channel, then, as the permeant ion (sodium) concentration is increased, the potency of the divalent blocker should decrease. In this chapter, a difference in  $K_i$  for calcium in reducing single channel currents at 250 and 75 mM sodium (symmetrical) suggested competition between calcium and sodium. As the concentration of sodium was increased, the apparent inhibition constant,  $K_i$ , was increased. However, the general characteristics of the voltage dependence of inward current block and the voltage-independent outward current block was retained. When divalent cation block was studied under a wider range of sodium ion concentrations (50-1000 mM), divalents were found to be less potent as sodium was increased. These alterations in current reduction were consistent with divalents competing with sodium for a common site located on the channel. In the presence of 10 mM calcium or 20 mM strontium, the channel's apparent affinity for sodium is decreased over 100 fold. However, the maximum current is slightly altered in the presence of divalents and appears to be dependent on voltage. These results suggest a form of competitive interactions between sodium and calcium however, the exact mechanism is, at present, unknown.

## 6. CALCIUM CHANNELS FROM RAT BRAIN IN PLANAR LIPID BILAYERS.

Voltage-dependent calcium channels from rat brain were incorporated into planar lipid bilayers (Nelson, French, Krueger, 1984 (12)). A membrane fraction from rat brain median eminence was used as a source of these channels because that region of the brain consists primarily of nerve terminals. Nerve terminals are thought to have a high concentration of calcium channels. These brain single calcium channels have the following properties:

1. Selectivity: These channels select for calcium, barium, strontium, manganese over sodium, potassium, chloride, lanthanum, cadmium.

2. Unit Conductance: Barium (8-9 pS) > Calcium, Strontium (5-6 pS) > Manganese (3-4 pS) as reported in Nelson et al. (31) and Nelson (32).

3. Voltage-dependence: Membrane depolarization increased the opening rate constant and decreased the closing rate constant (31). The relationship between the probability of the channel being open and voltage could be described by the Boltzmann equation with an "apparent gating charge" of 1.5-2.0 (32).

4. Ion-dependence of kinetics: It has been traditionally thought that the kinetics of an ion channel are independent of the nature of the ion carrying charge through the channel. A significant finding of our studies was that the nature of the permeant divalent cation not only affected the single channel conductance, but it also affected the channel's closing rate constant (i.e. mean open times), with the same order of potency, i.e. Barium > Calcium, Strontium > Manganese (31) (Figure 5). This observation suggests that competition by the permeant ions for common sites in the channel may regulate both the rate of ion passage through the channel and its closing rate (i.e. occupancy of a site in the channel by a permeant ion would impede channel closing). This suggestion is further supported by the observation that the addition of manganese to solution containing a more permeant ion both decreased the single channel conductance and closing rate constant (32).

5. Block of channel by inorganic calcium channel blockers: A variety of inorganic ions including cadmium, cobalt, nickel, manganese, and lanthanum can reduce calcium currents in many preparations. We found that these inorganic calcium channel blockers reduce the single channel current in a dose dependent manner, with the order of potency being lanthanum > cadmium > manganese (Figure 6) (32). The efficacy of block depended not only on the nature of the blocking ion but also the nature of the permeant ion. The block was consistent with one blocking ion binding to a site in the channel and thereby impeding ion flow through the channel.

Recent work reported in Nelson (13) has demonstrated that these brain calcium channels are also slightly permeable to  $Mn^{++}$  and that  $Mn^{++}$  can also act as a blocker of  $Sr^{++}$ ,  $Ca^{++}$ , and  $Ba^{++}$  permeation through the channels. As expected, of the divalent cations tested,  $Mn^{++}$  had the smallest single channel conductance (4 pS) but displayed the longest open dwell times. Evidence is also presented that permeant divalent cations must interact with at least two binding sites in traversing the channel pore.

# LITERATURE CITED

1. Krueger, B.K., R.W. Ratzliff, G.R. Strichartz, and M.P. Blaustein. Saxitoxin binding to synaptosomes, membranes, and solubilized binding sites from rat brain. J. Membrane Biol. 50: 287-310 (1979).
2. Krueger, B.K., J.F. Worley, and R.J. French. Single sodium channels from rat brain incorporated into planar lipid bilayer membranes. Nature 303: 172-175 (1983).
3. French, R.J., J.F. Worley, and B.K. Krueger. Voltage-dependent block by saxitoxin of sodium channels incorporated into planar lipid bilayers. Biophys. J. 45: 301-310 (1984).
4. Almers, W. and S.R. Levinson. Tetrodotoxin binding to normal and depolarized frog muscle and the conductance of a single sodium channel. J. Physiol. 252: 483-509 (1975).
5. Ulbricht, W. and H.-H. Wagner. The influence of pH on equilibrium effects of tetrodotoxin on myelinated nerve fibers of Rana esculenta. J. Physiol. 252: 159-184 (1975).
6. Ulbricht, W. and H.-H. Wagner. The influence of pH on the rate of tetrodotoxin action on myelinated nerve fibers. J. Physiol. 252: 185-202 (1975).
7. Baer, M., P.M. Best, and H. Reuter. Voltage-dependent action of tetrodotoxin in mammalian cardiac muscle. Nature 263: 344-345 (1976).
8. Cohen, I.S. and G.R. Strichartz. On the voltage-dependent action of tetrodotoxin. Biophys. J. 17: 275-279 (1977).
9. Cohen, C.J., B.P. Bean, T.J. Colatsky, and R.W. Tsien. Tetrodotoxin block of sodium channels in rabbit Purkinje fibers. Interactions between toxin binding and channel gating. J. Gen. Physiol. 78: 383-411 (1981).
10. Woodhull, A.M. Ionic blockage of sodium channels in nerve. J. Gen. Physiol. 61: 687-708 (1973).
11. French, R.J. and W.J. Adelman, Jr. Competition, saturation, and inhibition - ionic interactions shown by membrane ionic currents in nerve, muscle, and bilayer systems. Curr. Top. Membranes Transport 8: 161-207 (1976).
12. Reed, J. K., and M. A. Raftery. 1976. Properties of the tetrodotoxin binding component in plasma membranes isolated from Electrophorus Electricus. Biochemistry 15:944-953.
13. Hille, B. 1968. Charges and potentials at the nerve surface. Divalent ions and pH. J. Physiol. 51: 221-236.
14. Brodwick, M. S., and D. C. Eaton. Chemical modification of excitable membranes. In "Proteins in the Nervous System: Structure and Function." B. Haber, R. Perez-Polo, eds. Alan R. Liss, New York, pp. 51-72.
15. Sigworth, F. J., and B. C. Spelding. 1980. Chemical modification reduces the conductance of sodium channels in nerve. Nature (London). 282:293-295.



16. Spalding, B. C. 1980. Properties of toxin-resistant sodium channels produced by chemical modification in frog skeletal muscle. J. Physiol. 305:485-500.
17. Baker, P. F., and K. A. Robinson. 1977. TTX-resistant action potentials in crab nerve after treatment with Meerwein's Reagent. J. Physiol. 206:3-4P.
18. Yamamoto, D., J. Z. Yeh, and T. Marshashi. 1984. Voltage-dependent calcium block of normal and tetraethrin-modified single sodium channels. Biophys. J. 45:337-344.
19. Frankenhaeuser, B., and A. L. Hodgkin. 1957. The action of calcium on the electrical properties of squid axons. J. Physiol. 137:218-244.
20. Huang, L. -Y. M., M. Moran, and G. Ehrenstein. 1984. Gating kinetics of batrachotoxin-modified sodium channels in neuroblastoma cells determined from single-channel measurements. Biophys. J. 45: 313-322.
21. Moczydlowski, E., S. S. Garber, and C. Miller. 1984a. Batrachotoxin-activated  $Na^+$  channels in planar lipid bilayers. Competition of tetrodotoxin block by  $Na^+$ . J. Gen. Physiol. 84:665-686.
22. Hodgkin, A. L., and A. F. Huxley. 1952a. Currents carried by sodium and potassium ions through the membrane of the giant axon of Loligo. J. Physiol. 116:449-472.
23. Hille, B. 1975. Ionic selectivity, saturation, and block in sodium channels. A four-barrier model. J. Gen. Physiol. 66:535-560.
24. Begenisich, T., and D. Busath. 1981. Sodium flux ratio in voltage-clamped squid giant axon. J. Gen. Physiol. 77:489-502.
25. Begenisich, T. B., and M. D. Cahalan. 1980a. Sodium channel permeation in squid axons I: Reversal potential experiments. J. Physiol. 307:217-242.
26. Begenisich, T. B., and M. D. Cahalan. 1980b. Sodium channel permeation in squid axons II: Non-independence and current-voltage relations. J. Physiol. 307:243-257.
27. Bell, J. E., and C. Miller. 1984. Effects of phospholipid surface charge on ion conduction in the  $K^+$  channel of sarcoplasmic reticulum. Biophys. J. 45:279-287.
28. Apell, H. J., E. Bamberg, and P. Lauger. 1979. Effects of surface charge on the conductance of the gramicidin channel. Biochim. Biophys. Acta. 552:369-378.
29. Levinson, S. R., and J. C. Ellory. 1973. Molecular size of the tetrodotoxin site estimated by irradiation inactivation. Nature. (London). 245:122-123.
30. Hille, B., A. M. Woodhull, and B. I. Shapiro. 1975. Negative surface charge near the sodium channels of nerve: divalent ions, monovalent ions, and pH. Philos. Trans. R. Soc. London Ser. B. 270:301-3

- 31. Nelson, M.T., R.J. French, and B.K. Krueger. Single calcium channels from rat brain in planar lipid bilayers. Nature 308: 77-80 (1984).
- 32. Nelson, M.T. Interactions of divalent cations with single calcium channels from rat brain synaptosomes. J. Gen. Physiol. 87: 201-222 (1986).

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